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## Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 148 605 B2

(12)

## NEW EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the opposition decision:  
**23.12.1998 Bulletin 1998/52**

(51) Int Cl. 6: **C12N 15/00, C12P 21/02,**  
**C12N 1/00, C12N 5/00,**  
**C12Q 1/68, C07K 14/00,**  
**C07K 2/00, A61K 38/08**

(45) Mention of the grant of the patent:  
25.07.1990 Bulletin 1990/30

(21) Application number: 84308654.7

(22) Date of filing: 12.12.1984

#### (54) Production of erythropoietin

## Herstellung von Erythropoietin

## Production d'érythropoïétine

(84) Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

(30) Priority: 13.12.1983 US 561024  
21.02.1984 US 582185  
28.09.1984 US 655841  
30.11.1984 US 675298

(43) Date of publication of application:  
17.07.1985 Bulletin 1985/29

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- PROC. NATL. ACAD. SCI. USA, vol. 80, February 1983, pages 802-806; US. J. SINGER-SAM et al.: "Isolation of a cDNA clone for human X-linked 3-phosphoglycerate kinase by use of a mixture of synthetic oligo-deoxyribonucleotides as a detection probe."
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**Description****Background**

5 The present invention relates generally to the manipulation of genetic materials and, more particularly, to recombinant procedures making possible the production of polypeptides possessing part or all of the primary structural conformation.

**A. Manipulation Of Genetic Materials**

10 Genetic materials may be broadly defined as those chemical substances which program for and guide the manufacture of constituents of cells and viruses and direct the responses of cells and viruses. A long chain polymeric substance known as deoxyribonucleic acid (DNA) comprises the genetic material of all living cells and viruses except for certain viruses which are programmed by ribonucleic acids (RNA). The repeating units in DNA polymers are four different nucleotides, each of which consists of either a purine (adenine or guanine) or a pyrimidine (thymine or cytosine) bound to a deoxyribose sugar to which a phosphate group is attached. Attachment of nucleotides in linear polymeric form is by means of fusion of the 5' phosphate of one nucleotide to the 3' hydroxyl group of another. Functional DNA occurs in the form of stable double stranded associations of single strands of nucleotides (known as deoxyoligonucleotides), which associations occur by means of hydrogen bonding between purine and pyrimidine bases [i.e., "complementary" associations existing between adenine (A) and thymine (T) or guanine (G) and cytosine (C)]. By convention, nucleotides are referred to by the names of their constituent purine or pyrimidine bases, and the complementary associations of nucleotides in double stranded DNA (i.e., A—T and G—C) are referred to as "base pairs". Ribonucleic acid is a polynucleotide comprising adenine, guanine, cytosine and uracil (U), rather than thymine, bound to ribose and a phosphate group.

20 25 Most briefly put, the programming function of DNA is generally effected through a process wherein specific DNA nucleotide sequences (genes) are "transcribed" into relatively unstable messenger RNA (mRNA) polymers. The mRNA, in turn, serves as a template for the formation of structural, regulatory and catalytic proteins from amino acids. This mRNA "translation" process involves the operations of small RNA strands (tRNA) which transport and align individual amino acids along the mRNA strand to allow for formation of polypeptides in proper amino acid sequences. The mRNA "message", derived from DNA and providing the basis for the tRNA supply and orientation of any given one of the twenty amino acids for polypeptide "expression", is in the form of triplet "codons" — sequential groupings of three nucleotide bases. In one sense, the formation of a protein is the ultimate form of "expression" of the programmed genetic message provided by the nucleotide sequence of a gene.

30 35 "Promoter" DNA sequences usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

40 45 A focus of microbiological processing for the last decade has been the attempt to manufacture industrially and pharmaceutically significant substances using organisms which either do not initially have genetically coded information concerning the desired product included in their DNA, or (in the case of mammalian cells in culture) do not ordinarily express a chromosomal gene at appreciable levels. Simply put, a gene that specifies the structure of a desired polypeptide product is either isolated from a "donor" organism or chemically synthesized and then stably introduced into another organism which is preferably a self-replicating unicellular organism such as bacteria, yeast or mammalian cells in culture. Once this is done, the existing machinery for gene expression in the "transformed" or "transfected" microbial host cells operates to construct the desired product, using the exogenous DNA as a template for transcription of mRNA which is then translated into a continuous sequence of amino acid residues.

50 55 The art is rich in patent and literature publications relating to "recombinant DNA" methodologies for the isolation, synthesis, purification and amplification of genetic materials for use in the transformation of selected host organisms. U.S. Letters Patent No. 4,237,224 to Cohen, at al., for example, relates to transformation of unicellular host organisms with "hybrid" viral or circular plasmid DNA which includes selected exogenous DNA sequences. The procedures of the Cohen, at al. patent first involve manufacture of a transformation vector by enzymatically cleaving viral or circular plasmid DNA to form linear DNA strands. Selected foreign ("exogenous" or "heterologous") DNA strands usually including sequences coding for desired product are prepared in linear form through use of a similar enzymes. The linear viral or plasmid DNA is incubated with the foreign DNA in the presence of ligating enzymes capable of effecting a restoration process and "hybrid" vectors are formed which include the selected exogenous DNA segment "spliced"

into the viral or circular DNA plasmid.

Transformation of compatible unicellular host organisms with the hybrid vector results in the formation of multiple copies of the exogenous DNA in the host cell population. In some instances, the desired result is simply the amplification of the foreign DNA and the "product" harvested is DNA. More frequently, the goal of transformation is the expression by the host cells of the exogenous DNA in the form of large scale synthesis of isolatable quantities of commercially significant protein or polypeptide fragments coded for by the foreign DNA. See also, e.g., U.S. Letters Patent Nos. 4,264,731 (to Shine), 4,273,875 (to Manis), 4,293,652 (to Cohen), and European Patent Application 093,619, published November 9, 1983.

The development of specific DNA sequences for splicing into DNA vectors is accomplished by a variety of techniques, depending to a great deal on the degree of "foreignness" of the "donor" to the projected host and the size of the polypeptide to be expressed in the host. At the risk of over-simplification, it can be stated that three alternative principal methods can be employed: (1) the "isolation" of double-stranded DNA sequence from the genomic DNA of the donor; (2) the chemical manufacture of a DNA sequence providing a code for a polypeptide of interest; and (3) the *in vitro* synthesis of a double-stranded DNA sequence by enzymatic "reverse transcription" of mRNA isolated from donor cells. The last-mentioned methods which involve formation of a DNA "complement" of mRNA are generally referred to as "cDNA" methods.

Manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. DNA manufacturing procedures of co-owned, co-pending U.S. Patent Application Serial No. 483,451, by Alton, et al., (filed April 15, 1983 and corresponding to PCT US83/00605, published November 25, 1983 as WO83/04053), for example, provide a superior means for accomplishing such highly desirable results as: providing for the presence of alternate codons commonly found in genes which are highly expressed in the host organism selected for expression (e.g., providing yeast or *E. coli* "preference" codons); avoiding the presence of untranslated "intron" sequences (commonly present in mammalian genomic DNA sequences and mRNA transcripts thereof) which are not readily processed by prokaryotic host cells; avoiding expression of undesired "leader" polypeptide sequences commonly coded for by genomic DNA and cDNA sequences but frequently not readily cleaved from the polypeptide of interest by bacterial or yeast host cells; providing for ready insertion of the DNA in convenient expression vectors in association with desired promotor/regulator and terminator sequences; and providing for ready construction of genes for polypeptide fragments and analogs of the desired polypeptides.

When the entire sequence of amino acid residues of the desired polypeptide is not known, direct manufacture of DNA sequences is not possible and isolation of DNA sequences coding for the polypeptide by a cDNA method becomes the method of choice despite the potential drawbacks in ease of assembly of expression vectors capable of providing high levels of microbial expression referred to above. Among the standard procedures for isolating cDNA sequences of interest is the preparation of plasmid-borne cDNA "libraries" derived from reverse transcription of mRNA abundant in donor cells selected as responsible for high level expression of genes (e.g., libraries of cDNA derived from pituitary cells which express relatively large quantities of growth hormone products). Where substantial portions of the polypeptide's amino acid sequence are known, labelled, single-stranded DNA probe sequences duplicating a sequence putatively present in the "target" cDNA may be employed in DNA/DNA hybridization procedures carried out on cloned copies of the cDNA which have been denatured to single stranded form. [See, generally, the disclosure and discussions of the art provided in U.S. Patent No. 4,394,443 to Weissman, et al. and the recent demonstrations of the use of long oligonucleotide hybridization probes reported in Wallace, et al., *Nuc. Acids Res.*, 6, pp. 3543—3557 (1979), and Reyes, et al., *P.N.A.S. (U.S.A.)*, 79, pp. 3270—3274 (1982), and Jaye, et al., *Nuc. Acids Res.*, 11, pp. 2325—2335 (1983). See also, U.S. Patent No. 4,358,535 to Falkow, et al., relating to DNA/DNA hybridization procedures in effecting diagnosis; published European Patent Application Nos. 0070685 and 0070687 relating to light-emitting labels on single stranded polynucleotide probes; Davis, et al., "A Manual for Genetic Engineering, Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1980) at pp. 55—58 and 174—176, relating to colony and plaque hybridization techniques; and, New England Nuclear (Boston, Mass.) brochures for "Gene Screen" Hybridization Transfer Membrane materials providing instruction manuals for the transfer and hybridization of DNA and RNA, Catalog No. NEF-972.]

Among the more significant recent advances in hybridization procedures for the screening of recombinant clones is the use of labelled mixed synthetic oligonucleotide probes, each of which is potentially the complete complement of a specific DNA sequence in the hybridization sample including a heterogenous mixture of single stranded DNAs and RNAs. These procedures are acknowledged to be especially useful in the detection of cDNA clones derived from sources which provide extremely low amounts of mRNA sequences for the polypeptide of interest. Briefly put, use of stringent hybridization conditions directed toward avoidance of non-specific binding can allow, e.g., for the autoradiographic visualization of a specific cDNA clone upon the event of hybridization of the target DNA to that single probe within the mixture which is its complete complement. See generally, Wallace, et al., *Nuc. Acids Res.*, 9, pp. 879—897 (1981); Suggs, et al., *P.N.A.S. (U.S.A.)*, 78, pp. 6613—6617 (1981); Choo, et al., *Nature*, 299, pp. 178—180 (1982); Kurachi, et al., *P.N.A.S. (U.S.A.)*, 79, pp. 6461—6464 (1982); Ohkubo, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 2196—2200.

(1983); and Kornblihtt, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 3218-3222 (1983). In general, the mixed probe procedures of Wallace, et al. (1981), *supra*, have been expanded upon by various workers to the point where reliable results have reportedly been obtained in a cDNA clone isolation using a 32 member mixed "pool" of 16-base-long (16-mer) oligonucleotide probes of uniformly, varying DNA sequences together with a single 11-mer to effect a two-site "positive" confirmation of the presence of cDNA of interest. See, Singer-Sam, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 802-806 (1983).

The use of genomic DNA isolates is the least common of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures. This is especially true in the area of recombinant procedures directed to securing microbial expression of mammalian polypeptides and is due, principally to the complexity of mammalian genomic DNA. Thus, while reliable procedures exist for developing phage-borne libraries of genomic DNA of human and other mammalian species origins [See, e.g., Lawn, et al., *Cell*, 15, pp. 1157-1174 (1978) relating to procedures for generating a human genomic library commonly referred to as the "Maniatis Library"; Karn, et al., *P.N.A.S. (U.S.A.)*, 77, pp 5172-5176 (1980) relating to a human genomic library based on alternative restriction endonuclease fragmentation procedure; and Blattner, et al., *Science*, 196, pp. 161-169 (1977) describing construction of a bovine genomic library] there have been relatively few successful attempts at use of hybridization procedures in isolating genomic DNA in the absence of extensive foreknowledge of amino acid or DNA sequences. As one example, Fiddes, et al., *J. Mol. and App. Genetics*, 1, pp. 3-18 (1981) report the successful isolation of a gene coding for the alpha subunit of the human pituitary glycoprotein hormones from the Maniatis Library through use of a "full length" probe including a complete 621 base pair fragment of a previously-isolated cDNA sequence for the alpha subunit. As another example, Das, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 1531-1535 (1983) report isolation of human genomic clones for human HLA-DR using a 175 base pair synthetic oligonucleotide. Finally, Anderson, et al., *P.N.A.S. (U.S.A.)*, 80, pp. 6838-6842 (1983) report the isolation of genomic clone for bovine pancreatic trypsin inhibitor (BPTI) using a single probe 86 base pairs in length and constructed according to the known amino acid sequence of BPTI. The authors note a determination of poor prospects for isolating mRNA suitable for synthesis of a cDNA library due to apparent low levels of mRNA in initially targeted carotid gland and lung tissue sources and then address the prospects of success in probing a genomic library using a mixture of labelled probes, stating: "More generally, mixed-sequence oligodeoxyribonucleotide probes have been used to isolate protein genes of unknown sequence from cDNA libraries. Such probes are typically mixtures of 8-32 oligonucleotides, 14-17 nucleotides in length, representing every possible codon combination for a small stretch (5-6 residues) of amino acid sequence. Under stringent hybridization conditions that discriminate against incorrectly base-paired probes, these mixtures are capable of locating specific gene sequences in clone libraries of low-to-moderate complexity. Nevertheless, because of their short length and heterogeneity, mixed probes often lack the specificity required for probing sequences as complex as a mammalian genome. This makes such a method impractical for the isolation of mammalian protein genes when the corresponding mRNAs are unavailable." (Citations omitted).

There thus continues to exist a need in the art for improved methods for effecting the rapid and efficient isolation of cDNA clones in instances where little is known of the amino acid sequence of the polypeptide coded for and where "enriched" tissue sources of mRNA are not readily available for use in constructing cDNA libraries. Such improved methods would be especially useful if they were applicable to isolating mammalian genomic clones where sparse information is available concerning amino acid sequences of the polypeptide coded for by the gene sought.

#### 40 B. Erythropoietin As A Polypeptide Of Interest

Erythropoiesis, the production of red blood cells, occurs continuously throughout the human life span to offset cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available in the blood for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone, erythropoietin.

Erythropoietin, an acidic glycoprotein of approximately 34,000 dalton molecular weight, may occur in three forms:  $\alpha$ ,  $\beta$  and asialo. The  $\alpha$  and  $\beta$  forms differ slightly in carbohydrate components, but have the same potency, biological activity and molecular weight. The asialo form is an  $\alpha$  and  $\beta$  form with the terminal carbohydrate (sialic acid) removed. Erythropoietin is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is enough to stimulate replacement of red blood cells which are lost normally through aging.

The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused by loss of large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, erythropoietin will increase red blood cell production by stimulating the conversion of primitive precursor cells in the bone marrow into proerythroblasts which subsequently mature, synthesize hemoglobin and are released into the circulation as red blood

cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, erythropoietin in circulation is decreased.

See generally, Testa, et al., *Exp. Hematol.*, 8(Supp. 8), 144—152 (1980); Tong, et al., *J. Biol. Chem.*, 256(24), 1266—12672 (1981); Goldwasser, *J. Cell. Physiol.*, 110(Supp. 1), 133—135 (1982); Finch, *Blood*, 60(6), 1241—1246 (1982); Sztowski, et al., *Expt. Hematol.*, 8(Supp. 8), 52—64 (1980); Naughton, *Ann. Clin. Lab. Sci.*, 13(5), 432—438 (1983); Weiss, et al., *Am. J. Vet. Res.*, 44(10), 1832—1835 (1983); Lappin, et al., *Exp. Hematol.*, 11(7), 661—666 (1983); Baciu, et al., *Ann. N. Y. Acad. Sci.*, 414, 66—72 (1983); Murphy, et al., *Acta Haematologica Japonica*, 46(7), 1380—1396 (1983); Dessypris, et al., *Brit. J. Haematol.*, 56, 295—306 (1984); and, Emmanuel, et al., *Am. J. Physiol.* 247 (1 Pt 2), F168—76 (1984).

Because erythropoietin is essential in the process of red blood cell formation, the hormone has potential useful application in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. See, generally, Pennathur-Das, et al., *Blood*, 63(5), 1168—71 (1984) and Haddy, *Am. Jour. Ped. Hematol./Oncol.*, 4, 191—196, (1982) relating to erythropoietin in possible therapies for sickle cell disease, and Eschbach, et al. *J. Clin. Invest.*, 74(2), pp. 434—441, (1984), describing a therapeutic regimen for uremia sheep based on *in vivo* response to erythropoietin-rich plasma infusions and proposing a dosage of 10 U EPO/kg per day for 15—40 days as corrective of anemia of the type associated with chronic renal failure. See also, Krane, *Henry Ford Hosp. Med. J.*, 31 (3), 177—181 (1983).

It has recently been estimated that the availability of erythropoietin in quantity would allow for treatment each year of anemias of 1,600,000 persons in the United States alone. See, e.g., Morrison, "Bioprocessing in Space — an Overview", pp. 557—571 in The World Biotech Report 1984, Volume 2: USA, (Online Publications, New York, N.Y. 1984). Recent studies have provided a basis for projection of efficacy of erythropoietin therapy in a variety of disease states, disorders and state of hematologic irregularity; Vedovato, et al., *Acta Haematol.*, 71, 211—213 (1984) (beta-thalassemia); Vichinsky, et al., *J. Pediatr.* 105(1), 15—21 (1984) (cystic fibrosis); Cotes, et al., *Brit. J. Obstet. Gynaecol.* 90(4), 304—311 (1983) (pregnancy, menstrual disorders); Haga, et al., *Acta. Pediatr. Scand.*, 72, 827—831 (1983) (early anemia of prematurity); Claus-Walker, et al., *Arch. Phys. Med. Rehabil.*, 65, 370—374 (1984) (spinal cord injury); Dunn, et al., *Eur. J. Appl. Physiol.*, 52, 178—182 (1984), (space flight); Miller, et al., *Brit J. Haematol.*, 52., 545—590 (1982) (acute blood loss); Udupa, et al., *J. Lab. Clin. Med.*, 103(4), 574—580 and 581—588 (1984); and Lipschitz, et al., *Blood*, 63(3), 502—509 (1983) (aging); and Dainiak, et al., *Cancer*, 51(6), 1101—1106 (1983) and Schwartz, et al., *Otolaryngol.*, 109, 269—272 (1983) (various neoplastic disease states accompanied by abnormal erythropoiesis).

Prior attempts to obtain erythropoietin in good yield from plasma or urine have proven relatively unsuccessful. Complicated and sophisticated laboratory techniques are necessary and generally result in the collection of very small amounts of impure and unstable extracts containing erythropoietin.

U.S. Letters Patent No. 3,033,753 describes a method for partially purifying erythropoietin from sheep blood plasma which provides low yields of a crude solid extract containing erythropoietin.

Initial attempts to isolate erythropoietin from urine yielded unstable, biologically inactive preparations of the hormone. U.S. Letters Patent No. 3,865,801 describes a method of stabilizing the biological activity of a crude substance containing erythropoietin recovered from urine. The resulting crude preparation containing erythropoietin purportedly retains 90% of erythropoietin activity, and is stable.

Another method of purifying human erythropoietin from urine of patients with aplastic anemia is described in Miyake, et al., *J. Biol. Chem.*, Vol. 252, No. 15 (August 10, 1977), pp. 5558—5564. This seven-step procedure includes ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, and yields a pure erythropoietin preparation with a potency of 70,400 units/mg of protein in 21% yield.

U.S. Letters Patent No. 4,397,840 to Takezawa, et al., describes methods of preparing "an erythropoietin product" from healthy human urine specimens with weakly basic ion exchangers and proposes that the low molecular weight products obtained "have no inhibitory effects against erythropoietin."

U.K. Patent Application No. 2,085,887 by Sugimoto, et al., published May 6, 1982, describes a process for the production of hybrid human lymphoblastoid cells, reporting production levels ranging from 3 to 420 Units of erythropoietin per ml of suspension of cells (distributed into the cultures after mammalian host propagation containing up to  $10^7$  cells per ml. At the highest production levels asserted to have been obtained, the rate of erythropoietin production could be calculated to be 40 to about 4,000 Units/ $10^6$  cells/48 hours in *in vitro* culture following transfer of cells from *in vivo* propagation systems. (See also the equivalent U.S. Letters Patent No. 4,377,513.) Numerous proposals have been made for isolation of erythropoietin from tissue sources, including neoplastic cells, but the yields have been quite low. See, e.g., Jelkman, et al., *Expt. Hematol.*, 11(7), 581—588 (1983); Tambourin, et al., *P.N.A.S. (U.S.A.)*, 80, 6269—6273 (1983); Katsuoka, et al., *Gann*, 74, 534—541 (1983); Hagiwara, et al., *Blood*, 63(4), 828—835 (1984); and Choppin, et al., *Blood*, 64(2), 341—347 (1984).

Other isolation techniques utilized to obtain purified erythropoietin involve immunological procedures. A polyclonal, serum-derived antibody directed against erythropoietin is developed by injecting an animal, preferably a rat or rabbit, with human erythropoietin. The injected human erythropoietin is recognized as a foreign antigenic substance by the

immune system of the animal and elicits production of antibodies against the antigen. Differing cells responding to stimulation by the antigenic substance produce and release into circulation antibodies slightly different from those produced by other responding cells. The antibody activity remains in the serum of the animal when its blood is extracted. While unpurified serum or antibody preparations purified as a serum immunoglobulin G fraction may then be used in assays to detect and complex with human erythropoietin, the materials suffer from a major disadvantage. This serum antibody, composed of all the different antibodies produced by individual cells, is polyclonal in nature and will complex with components in crude extracts other than erythropoietin alone.

Of interest to the background of the present invention are recent advances in the art of developing continuous cultures of cells capable of producing a single species of antibody which is specifically immunologically reactive with a single antigenic determinant of a selected antigen. See, generally, Chisholm, *High Technology*, Vol. 3, No. 1, 57—63 (1983). Attempts have been made to employ cell fusion and hybridization techniques to develop "monoclonal" antibodies to erythropoietin and to employ these antibodies in the isolation and quantitative detection of human erythropoietin. As one example, a report of the successful development of mouse-mouse hybridoma cell lines secreting monoclonal antibodies to human erythropoietin appeared in abstract form in Lee-Huang, Abstract No. 1463 of *Fed. Proc.*, 41, 520 (1982). As another example, a detailed description of the preparation and use of a monoclonal, anti-erythropoietin antibody appears in Weiss, et al., *P.N.A.S. (U.S.A.)*, 79, 5465—5469 (1982). See also, Sasaki, *Biomed. Biochim. Acta.*, 42(11/12), S202—S206 (1983); Yanagawa, et al., *Blood*, 64(2), 357—364 (1984); Yanagawa, et al., *J. Biol. Chem.*, 259(5), 2707—2710 (1984); and U. S Letters Patent No. 4,465,624.

Also of interest to the background of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically active animals. See, e.g., Lerner, et al., *Cell*, 23, 309—310 (1981); Ross, et al., *Nature*, 294, 654—656 (1981); Walter, et al., *P.N.A.S. (U.S.A.)*, 77, 5197—5200 (1980); Lerner, et al., *P.N.A.S. (U.S.A.)*, 78, 3403—3407 (1981); Walter, et al., *P.N.A.S. (U.S.A.)*, 78, 4882—4886 (1981); Wong, et al., *P.N.A.S. (U.S.A.)*, 78, 7412—7416 (1981); Green, et al., *Cell*, 28, 477—487 (1982); Nigg, et al., *P.N.A.S. (U.S.A.)*, 79, 5322—5326 (1982); Baron, et al., *Cell*, 28, 395—404 (1982); Dreesman, et al., *Nature*, 295, 158—160 (1982); and Lerner, *Scientific American*, 248, No. 2, 66—74 (1983). See, also, Kaiser, et al., *Science*, 223, pp. 249—255 (1984) relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation. The above studies relate, of course, to amino acid sequences of proteins other than erythropoietin, a substance for which no substantial amino acid sequence information has been published. In co-owned, co-pending U.S. Patent Application Serial No. 463,724, filed February 4, 1983, by J. Egrie, published August 22, 1984 as European Patent Application No. 0 116 446, there is described a mouse-mouse hybridoma cell line (A.T.C.C. No. HB8209) which produces a highly specific monoclonal, anti-erythropoietin antibody which is also specifically immunoreactive with a polypeptide comprising the following sequence of amino acids:

following sequence of amino acids:

40 NH<sub>2</sub>-Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-COOH.

The polypeptide sequence is one assigned to the first twenty amino acid residues of mature human erythropoietin isolated according to the method of Miyake, et al., *J. Biol. Chem.*, 252, 5558—5564 (1977) and upon which amino acid analysis was performed by the gas phase sequencer (Applied Biosystems, Inc.) according to the procedure of Hewick, M., et al., *J. Biol. Chem.*, 256, 7990—7997 (1981). See, also, Sue, et al., *Proc. Nat. Acad. Sci. (USA)*, 80, pp. 3651—3655 (1983) relating to development of polyclonal antibodies against a synthetic 26-mer based on a differing amino acid sequence, and Sytowski, et al., *J. Immunol. Methods*, 69, pp. 181—186 (1984).

While polyclonal and monoclonal antibodies as described above provide highly useful materials for use in immunoassays for detection and quantification of erythropoietin and can be useful in the affinity purification of erythropoietin, it appears unlikely that these materials can readily provide for the large scale isolation of quantities of erythropoietin from mammalian sources sufficient for further analysis, clinical testing and potential wide-ranging therapeutic use of the substance in treatment of, e.g., chronic kidney disease wherein diseased tissues fail to sustain production of erythropoietin. It is consequently projected in the art that the best prospects for fully characterizing mammalian erythropoietin and providing large quantities of it for potential diagnostic and clinical use involve successful application of recombinant procedures to effect large scale microbial synthesis of the compound.

While substantial efforts appear to have been made in attempted isolation of DNA sequences coding for human and other mammalian species erythropoietin, none appear to have been successful. This is due principally to the

scarcity of tissue sources, especially human tissue sources, enriched in mRNA such as would allow for construction of a cDNA library from which a DNA sequence coding for erythropoietin might be isolated by conventional techniques. Further, so little is known of the continuous sequence of amino acid residues of erythropoietin that it is not possible to construct, e.g., long polynucleotide probes readily capable of reliable use in DNA/DNA hybridization screening of cDNA and especially genomic DNA libraries. Illustratively, the twenty amino acid sequence employed to generate the above-named monoclonal antibody produced by A.T.C.C. No. HB8209 does not admit to the construction of an unambiguous, 5 60 base oligonucleotide probe in the manner described by Anderson, et al., *supra*. It is estimated that the human gene for erythropoietin may appear as a "single copy gene" within the human genome and, in any event, the genetic material coding for human erythropoietin is likely to constitute less than 0.00005% of total human genomic DNA which would be present in a genomic library.

10 To date, the most successful of known reported attempts at recombinant-related methods to provide DNA sequences suitable for use in microbial expression of isolatable quantities of mammalian erythropoietin have fallen far short of the goal. As an example, Farber, et al. *Exp. Hematol.*, 11, Supp. 14, Abstract 101 (1983) report the extraction of mRNA from kidney tissues of phenylhydrazine-treated baboons and the injection of the mRNA into *Xenopus laevis* oocytes with the rather transitory result of *in vitro* production of a mixture of "translation products" which included among them 15 displaying biological properties of erythropoietin. More recently, Farber, et al., *Blood*, 62, No. 5, Supp. No. 1, Abstract 392, at page 122a (1983) reported the *in vitro* translation of human kidney mRNA by frog oocytes. The resultant translation product mixture was estimated to include on the order of 220 mU of a translation product having the activity of 20 erythropoietin per microgram of injected mRNA. While such levels of *in vitro* translation of exogenous mRNA coding for erythropoietin were acknowledged to be quite low (compared even to the prior reported levels of baboon mRNA translation into the sought-for product) it was held that the results confirm the human kidney as a site of erythropoietin expression, allowing for the construction of an enriched human kidney cDNA library from which the desired gene might be isolated. [See also, Farber, *Clin. Res.*, 31(4), 769A (1983).]

25 Since the filing of U.S. Patent Application Serial Nos. 561,024 and 582,185, there has appeared a single report of the cloning and expression of what is asserted to have been human erythropoietin cDNA in *E. coli*. Briefly put, a number of cDNA clones were inserted into *E. coli* plasmids and  $\beta$ -lactamase fusion products were noted to be immunoreactive with a monoclonal antibody to an unspecified "epitope" of human erythropoietin. See, Lee-Huang, *Proc. Nat. Acad. Sci. (USA)*, 81, pp. 2708-2712 (1984).

30 the genetic material coding for human erythropoietin is likely to constitute less than 0.00005% of total human genomic DNA which would be present in a genomic library.

To date, the most successful of known reported attempts at recombinant-related methods to provide DNA sequences suitable for use in microbial expression of isolatable quantities of mammalian erythropoietin have fallen far short of the goal. As an example, Farber, et al. *Exp. Hematol.*, 11, Supp. 14 Abstract 101 (1983) report the extraction of mRNA from kidney tissues of phenylhydrazine-treated baboons and the injection of the mRNA into *Xenopus laevis* oocytes with the rather transitory result of *in vitro* production of a mixture of "translation products" which included among them 35 displaying biological properties of erythropoietin. More recently, Farber, et al., *Blood*, 62, No. 5, Supp. No. 1, Abstract 392, at page 122a (1983) reported the *in vitro* translation of human kidney mRNA by frog oocytes. The resultant translation product mixture was estimated to include on the order of 220 mU of a translation product having the activity of 40 erythropoietin per microgram of injected mRNA. While such levels of *in vitro* translation of exogenous mRNA coding for erythropoietin were acknowledged to be quite low (compared even to the prior reported levels of baboon mRNA translation into the sought-for product) it was held that the results confirm the human kidney as a site of erythropoietin expression, allowing for the construction of an enriched human kidney cDNA library from which the desired gene might be isolated. [See also, Farber, *Clin. Res.*, 31(4), 769A (1983).]

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#### 50 Brief Summary

The present invention provides:-

55 1. A DNA sequence for use in securing expression in a prokaryotic or eucaryotic host cell of a polypeptide product having at least part of the primary structural confirmation of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells and to increase hemoglobin synthesis or iron uptake, said DNA sequence selected from the group consisting of:

- (a) the DNA sequences set out in Tables V and VI or their complementary strands;
- (b) DNA sequences which hybridize under stringent conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and
- 5 (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).
- 2. A DNA sequence according to point 1 encoding human erythropoietin.
- 10 3. A cDNA sequence according to point 1 being a monkey species erythropoietin coding DNA sequence.
- 4. A DNA sequence according to point 3 and including the protein coding region set forth in Table V.
- 15 5. A genomic DNA sequence according to point 1 or 2.
- 6. A human species erythropoietin coding DNA sequence according to point 5.
- 7. A DNA sequence according to point 6 and including the protein coding region set forth in Table VI.
- 20 8. A DNA sequence according to point 1 or 2, covalently associated with a detectable label substance.
- 9. A DNA sequence according to point 8, wherein the detectable label is a radiolabel.
- 25 10. A single-strand DNA sequence according to point 8 or 9.
- 11. A DNA sequence according to point 1, coding for [Phe<sup>15</sup>]hEPO, [Phe<sup>49</sup>]hEPO, [Phe<sup>145</sup>]hEPO, [His<sup>7</sup>]hEPO, [Asn<sup>2</sup> des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO, [des-Thr<sup>16</sup>] through Arg<sup>166</sup>]hEPO, or [Δ27-55]hEPO.
- 30 12. A prokaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to any one of points 1, 2, 3, 6, 7 and 8, in a manner allowing the host cell to express said polypeptide product.
- 13. A transformed or transfected host cell according to point 12 which host cell is capable of glycosylating said polypeptide.
- 35 14. A transformed or transfected mammalian host cell according to point 13.
- 15. A transformed or transfected COS cell according to point 13.
- 40 16. A transformed or transfected CHO cell according to point 13.
- 17. A biologically functional circular plasmid or viral DNA vector including a DNA sequence according to any one of points 1, 2, 3, 5, 6, 7, or 11.
- 45 18. A prokaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to point 17.
- 19. A recombinant polypeptide having part or all of the primary structural conformation of human or monkey erythropoietin as set forth in Table VI or Table V or any allelic variant or derivative thereof possessing the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells to increase hemoglobin synthesis or iron uptake and characterized by being the product of eucaryotic expression of an exogenous DNA sequence and which has higher molecular weight by SDS-PAGE from erythropoietin isolated from urinary sources.
- 50 20. A glycoprotein polypeptide according to point 19 having an average carbohydrate composition which differs from that of human erythropoietin isolated from urinary sources.
- 21. A polypeptide according to point 19 or 20 wherein the exogenous sequence is a cDNA sequence.

22. A polypeptide according to point 19 or 20 wherein the exogenous DNA sequence is a genomic DNA sequence.

23. A polypeptide according to point 19 or 20 wherein the exogenous DNA sequence is carried on an autonomously replicating circular DNA plasmid or viral vector.

5

24. A polypeptide according to any one of points 19 to 23 further characterized by being covalently associated with a detectable label substance.

10

25. A polypeptide according to point 24, wherein said detectable label is a radiolabel.

15

26. A polypeptide product of the expression in a eucaryotic host cell of a DNA sequence according to any of points 1, 2, 3, 5, 6 and 7.

20

27. A process for production of a polypeptide having at least part of the primary structural conformation of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells and to increase hemoglobin synthesis or iron uptake, which process is characterized by culturing under suitable nutrient conditions a procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to any of points 1, 2, 3, 5, 6 and 7 in a manner allowing the host cell to express said polypeptide; and optionally isolating the desired polypeptide product of the expression of the DNA sequence.

25

28. A process according to point 27, characterized by culturing a host cell of any one of points 12 to 16.

25

29. A process according to point 27 or 28 for production of a polypeptide of any one of points 19 to 23 and 26.

30

30. A pharmaceutical composition comprising a polypeptide produced in accordance with the process of point 27, 28 or 29 and a pharmaceutically acceptable diluent, adjuvant or carrier.

35

31. A pharmaceutical composition according to point 30, comprising a polypeptide of any one of points 19 to 23 and 26.

35

Vertebrate (e.g., COS-1 and CHO) cells provided by the present invention comprise the first cells ever available which can be propagated *in vitro* continuously and which upon growth in culture are capable of producing in the medium of their growth in excess of 100U (preferably in excess of 500U and most preferably in excess of 1,000 to 5,000U) of erythropoietin per 10<sup>6</sup> cells in 48 hours as determined by radioimmunoassay.

40

Also provided by the present invention are synthetic polypeptides wholly or partially duplicative of continuous sequences of erythropoietin amino acid residues which are herein for the first time elucidated. These sequences, by virtue of sharing primary, secondary or tertiary structural and conformational characteristics with naturally-occurring erythropoietin may possess biological activity and/or immunological properties in common with the naturally-occurring product such that they may be employed as biologically active or immunological substitutes for erythropoietin in therapeutic and immunological processes.

Illustrating the present invention are cloned DNA sequences of monkey and human species origins and polypeptide sequences suitably deduced therefrom which represent, respectively, the primary structural conformation of erythropoietins of monkey and human species origins.

45

Also provided by the present invention are novel biologically functional viral and circular plasmid DNA vectors incorporating DNA sequences of the invention and microbial (e.g., bacterial, yeast and mammalian cell) host organisms stably transformed or transfected with such vectors. Correspondingly provided by the invention are novel methods for the production of useful polypeptides comprising cultured growth of such transformed or transfected microbial hosts under conditions facilitative of large scale expression of the exogenous, vector-borne DNA sequences and isolation of the desired polypeptides from the growth medium, cellular lysates or cellular membrane fractions.

Isolation and purification of microbially expressed polypeptides provided by the invention may be by conventional means including, e.g., preparative chromatographic separations and immunological separations involving monoclonal and/or polyclonal antibody preparations.

50

Having herein elucidated the sequence of amino acid residues of erythropoietin, the present invention provides for the total and/or partial manufacture of DNA sequences coding for erythropoietin and including such advantageous characteristics as incorporation of codons "preferred" for expression by selected non-mammalian hosts, provision of sites for cleavage by restriction endonuclease enzymes and provision of additional initial, terminal or intermediate DNA sequences which facilitate construction of readily expressed vectors. The Examples of the present invention provide

for manufacture (and development by site specific mutagenesis of monkey cDNA and genomic DNA) of DNA sequences coding for microbial expression of polypeptide analogs or derivatives of erythropoietin which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for EPO and/or substitution analogs wherein one or more residues specified are replaced by other residues and/or addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptide); and which share some or all the properties of naturally-occurring forms.

5 Novel DNA sequences of the invention include all sequences useful in securing expression in prokaryotic or eukaryotic host cells of polypeptide products having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin which are comprehended by: (a) the DNA sequences set out in Tables V and VI herein or their complementary strands; (b) DNA sequences which hybridize (under hybridization conditions such as illustrated herein or more stringent conditions) to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in part (b) are genomic DNA sequences encoding allelic variant forms of monkey and human erythropoietin and/or encoding other mammalian species of erythropoietin. Specifically comprehended by part (c) are manufactured DNA sequences encoding EPO, EPO fragments and EPO analogs which DNA sequences may incorporate codons facilitating translation of messenger RNA in non-vertebrate hosts.

10 Comprehended by the present invention is that class of polypeptides coded for by portions of the DNA complement to the top strand human genomic DNA sequence of Table VI herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., *Nucleic Acids Research*, 12, pp. 5049-5059 (1984).

15 Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable pharmaceutically acceptable diluent(s), adjuvant(s) and/or carrier(s) which allow for provision of erythropoietin therapy, especially in the treatment of anemic disease states and most especially such anemic states as attend chronic renal failure.

20 Polypeptide products of the invention may be "labelled" by covalent association with a detectable marker substance (e.g., radiolabelled with  $^{125}\text{I}$ ) to provide reagents useful in detection and quantification of erythropoietin in solid tissue and fluid samples such as blood or urine. DNA products of the invention may also be labelled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in DNA hybridization processes to locate the erythropoietin gene position and/or the position of any related gene family in the human, monkey and other mammalian species chromosomal map. They can also be used for identifying the erythropoietin gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders.

25 As hereinafter described in detail, the present invention further provides significant improvements in methods for detection of a specific single stranded polynucleotide of unknown sequence in a heterogeneous cellular or viral sample including multiple single-stranded polynucleotides where

30

- (a) a mixture of labelled single-stranded polynucleotide probes is prepared having uniformly varying sequences of bases, each of said probes being potentially specifically complementary to a sequence of bases which is putatively unique to the polynucleotide to be detected,
- (b) the sample is fixed to a solid substrate,
- (c) the substrate having the sample fixed thereto is treated to diminish further binding of polynucleotides thereto except by way of hybridization to polynucleotides in said sample,
- (d) the treated substrate having the sample fixed thereto is transiently contacted with said mixture of labelled probes under conditions facilitative of hybridization only between totally complementary polynucleotides, and
- (e) the specific polynucleotide is detected by monitoring for the presence of a hybridization reaction between it and a totally complementary probe within said mixture of labelled probes, as evidenced by the presence of a higher density of labelled material on the substrate at the locus of the specific polynucleotide in comparison to a background density of labelled material resulting from non-specific binding of labelled probes to the substrate.

35 The procedures are especially effective in situations dictating use of 64, 128, 256, 512, 1024 or more mixed polynucleotide probes having a length of 17 to 20 bases in DNA/DNA or RNA/RNA or DNA/RNA hybridizations.

40 As described *infra*, the above-noted improved procedures have illustratively allowed for the identification of cDNA clones coding for erythropoietin of monkey species origins within a library prepared from anemic monkey kidney cell mRNA. More specifically, a mixture of 128 uniformly varying 20-mer probes based on amino acid sequence information derived from sequencing fractions of human erythropoietin was employed in colony hybridization procedures to identify seven "positive" erythropoietin monkey cDNA clones within a total of 200,000 colonies. Even more remarkably, practice

45 of the improved procedures of the invention have allowed for the rapid isolation of three positive clones from within a screening of 1,500,000 phage plaques constituting a human genomic library. This was accomplished through use of the above-noted mixture of 128 20-mer probes together with a second set of 128 17-mer probes based on amino acid analysis of a different continuous sequence of human erythropoietin.

The above-noted illustrative procedures constitute the first known instance of the use of multiple mixed oligonucleotide probes in DNA/DNA hybridization processes directed toward isolation of mammalian genomic clones and the first known instance of the use of a mixture of more than 32 oligonucleotide probes in the isolation of cDNA clones.

5 Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illustrations of the practice of the invention in its presently preferred embodiments.

#### Detailed Description

10 According to the present invention, DNA sequences encoding part or all of the polypeptide sequence of human and monkey species erythropoietin (hereinafter, at times, "EPO") have been isolated and characterized. Further, the monkey and human origin DNA has been made the subject of eucaryotic and procaryotic expression providing isolatable quantities of polypeptides displaying biological (e.g., immunological) properties of naturally-occurring EPO as well as both *in vivo* and *in vitro* biological activities of EPO.

15 The DNA of monkey species origins was isolated from a cDNA library constructed with mRNA derived from kidney tissue of a monkey in a chemically induced anemic state and whose serum was immunologically determined to include high levels of EPO compared to normal monkey serum. The isolation of the desired cDNA clones containing EPO encoding DNA was accomplished through use of DNA/DNA colony hybridization employing a pool of 128 mixed, radiolabelled, 20-mer oligonucleotide probes and involved the rapid screening of 200,000 colonies. Design of the oligonucleotide probes was based on amino acid sequence information provided by enzymatic fragmentation and sequencing a small sample of human EPO.

20 The DNA of human species origins was isolated from a human genomic DNA library. The isolation of clones containing EPO-encoding DNA was accomplished through DNA/DNA plaque hybridization employing the above-noted pool of 128 mixed 20-mer oligonucleotide probes and a second pool of 128 radiolabelled 17-mer probes whose sequences were based on amino acids sequence information obtained from a different enzymatic human EPO fragment.

25 Positive colonies and plaques were verified by means of dideoxy sequencing of clonal DNA using a subset of 16 sequences within the pool of 20-mer probes and selected clones were subjected to nucleotide sequence analysis resulting in deduction of primary structural conformation of the EPO polypeptides encoded thereby. The deduced polypeptide sequences displayed a high degree of homology to each other and to a partial sequence generated by amino acid analysis of human EPO fragments.

30 A selected positive monkey cDNA clone and a selected positive human genomic clone were each inserted in a "shuttle" DNA vector which was amplified in *E.coli* and employed to transfect mammalian cells in culture. Cultured growth of transfected host cells resulted in culture medium supernatant preparations estimated to contain as much as 3000 mU of EPO per ml of culture fluid.

35 The following examples are presented by way of illustration of the invention and are specifically directed to procedures carried out prior to identification of EPO encoding monkey cDNA clones and human genomic clones, to procedures resulting in such identification, and to the sequencing, development of expression systems and immunological verification of EPO expression in such systems.

40 More particularly, Example 1 is directed to amino acid sequencing of human EPO fragments and construction of mixtures of radiolabelled probes based on the results of this sequencing. Example 2 is generally directed to procedures involved in the identification of positive monkey cDNA clones and thus provides information concerning animal treatment and preliminary radioimmunoassay (RIA) analysis of animal sera. Example 3 is directed to the preparation of the cDNA library, colony hybridization screening and verification of positive clones, DNA sequencing of a positive cDNA clone and the generation of monkey EPO polypeptides primary structural conformation (amino acid sequence) information. Example 4 is directed to procedures involved in the identification of positive human genomic clones and thus provides information concerning the source of the genomic library, plaque hybridization procedures and verification of positive clones. Example 5 is directed to DNA sequencing of a positive genomic clone and the generation of human EPO polypeptide amino acid sequence information including a comparison thereof to the monkey EPO sequence information. Example 6 is directed to procedures for construction of a vector incorporating EPO-encoding DNA derived from a positive monkey cDNA clone, the use of the vector for transfection of COS-1 cells and cultured growth of the transfected cells. Example 7 is directed to procedures for construction of a vector incorporating EPO-encoding DNA derived from a positive human genomic clone, the use of the vector for transfection of COS-1 cells and the cultured growth of the transfected cells. Example 8 is directed to immunoassay procedures performed on media supernatants obtained from the cultured growth of transfected cells according to Example 6 and 7. Example 9 is directed to *in vitro* and *in vivo* biological activity of microbially expressed EPO of Examples 6 and 7.

45 Example 10 is directed to a development of mammalian host expression systems for monkey species EPO cDNA and human species genomic DNA involving Chinese hamster ovary ("CHO") cells and to the immunological and biological activities of products of these expression systems as well as characterization of such products. Example 11 is

directed to the preparation of manufactured genes encoding human species EPO and EPO analogs, which genes include a number of preference codons for expression in *E. coli* and yeast host cells, and to expression systems based thereon. Example 12 relates to the immunological and biological activity profiles of expression products of the systems of Example II.

5

## Example 1

## A. Human EPO Fragment Amino Acid Sequencing

10 Human EPO was isolated from urine and subjected to tryptic digestion resulting in the development and isolation of 17 discrete fragments in quantities approximating 100—150 picomoles.

15 Fragments were arbitrarily assigned numbers and were analyzed for amino acid sequence by microsequence analysis using a gas phase sequencer (Applied Biosystems) to provide the sequence information set out in Table I, below, wherein single letter codes are employed and "X" designates a residue which was not unambiguously determined.

TABLE I

20

<u>Fragment No.</u>	<u>Sequence Analysis Result</u>
T4a	A-P-P-R
T4b	G-K-L-K
T9	A-L-G-A-Q-K
T13	V-L-E-R
T16	A-V-S-G-L-R
T18	L-F-R
T21	K-L-F-R
T25	Y-L-L-E-A-K
T26a	L-I-C-D-S-R
T26b	L-Y-T-G-E-A-C-R
T27	T-I-T-A-D-T-F-R
T28	E-A-I-S-P-P-D-A-A-M-A-A-P-L-R
T30	E-A-E-X-I-T-T-G-X-A-E-H-X-S-L- N-E-X-I-T-V-P
T31	V-Y-S-N-F-L-R
T33	S-L-T-T-L-L-R
T35	V-N-F-Y-A-W-K
T38	G-Q-A-L-L-V-X-S-S-Q-P-W- E-P-L-Q-L-H-V-D-K

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## B. Design and Construction of Oligonucleotide Probe Mixtures

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The amino acid sequences set out in Table I were reviewed in the context of the degeneracy of the genetic code for the purpose of ascertaining whether mixed probe procedures could be applied to DNA/DNA hybridization procedures on cDNA and/or genomic DNA libraries. This analysis revealed that within Fragment No. T35 there existed a series of 7 amino acid residues (Val-Asn-Phe-Tyr-Ala-Trp-Lys) which could be uniquely characterized as encoded for by one of

128 possible DNA sequences spanning 20 base pairs. A first set of 128 20-mer oligonucleotides was therefore synthesized by standard phosphoamidite methods (See, e.g., Beaucage, et al., *Tetrahedron Letters*, 22, pp. 1859—1862 (1981) on a solid support according to the sequence set out in Table II, below.

5

TABLE II

	<u>Residue</u>	<u>- Val</u>	<u>- Asn</u>	<u>Phe</u>	<u>Tyr</u>	<u>Ala</u>	<u>Trp</u>	<u>Lys</u>	
10	3'	CAA	TTG	AAG	ATG	CGA	ACC	TT	- 5'
		T	A	A	A	T			
		G				G			
		C				C.			

15

Further analysis revealed that within fragment No. T38 there existed a series of 6 amino acid residues (Gln-Pro-Trp-Glu-Pro-Leu) on the basis of which there could be prepared a pool of 128 mixed oligonucleotide 17-mer probes as set out in Table III below.

20

TABLE III

	<u>Residue</u>	<u>- Gln</u>	<u>Pro</u>	<u>Trp</u>	<u>Glu</u>	<u>Pro</u>	<u>Leu</u>	
25	3'	GTT	GGA	ACC	CTT	GGA	GA	- 5'
		C	T		C	T	A	
		G				G		
		C				C		

30

Oligonucleotide probes were labelled at the 5' end with gamma —  $^{32}\text{P}$ —ATP, 7500—8000 Ci/mmol (ICN) using T, polynucleotide kinase (NEN).

## Example 2

## 35 A. Monkey Treatment Procedures and RIA Analysis

Female Cynomolgus monkeys *Macaca fascicularis* (2.5—3 kg, 1.5—2 years old) were treated subcutaneously with a pH 7.0 solution of phenylhydrazine hydrochloride at a dosage level of 12.5 mg/kg on days 1, 3 and 5. The hematocrit was monitored prior to each injection. On day 7, or whenever the hematocrit level fell below 25% of the initial level, serum and kidneys were harvested after administration of 25 mg/kg doses of ketamine hydrochloride. Harvested materials were immediately frozen in liquid nitrogen and stored at -70°C.

## B. RIA for EPO

45 Radioimmunoassay procedures applied for quantitative detection of EPO in samples were conducted according to the following procedures:

An erythropoietin standard or unknown sample was incubated together with antiserum for two hours at 37°C. After the two hour incubation, the sample tubes were cooled on ice,  $^{125}\text{I}$ -labelled erythropoietin was added, and the tubes were incubated at 0°C for at least 15 more hours. Each assay tube contained 500  $\mu\text{l}$  of incubation mixture consisting of 50  $\mu\text{l}$  of diluted immune sera, 10,000 cpm of  $^{125}\text{I}$ -erythropoietin, 5  $\mu\text{l}$  trasylo and 0—250  $\mu\text{l}$  of either EPO standard or unknown sample, with PBS containing 0.1% BSA making up the remaining volume. The antiserum used was the second test bleed of a rabbit immunized with a 1% pure preparation of human urinary erythropoietin. The final antiserum dilution on the assay was adjusted so that the antibody-bound  $^{125}\text{I}$ -EPO did not exceed 10—20% of the input total counts. In general, this corresponded to a final antiserum dilution of from 1:50,000 to 1:100,000.

55 The antibody-bound  $^{125}\text{I}$ -erythropoietin was precipitated by the addition of 150  $\mu\text{l}$  Staph A. After a 40 min. incubation, the samples were centrifuged and the pellets were washed two times with 0.75 ml 10 mM Tris-HCl pH 8.2 containing 0.15M NaCl, 2mM EDTA, and 0.05% Triton X-100. The washed pellets were counted in a gamma counter to determine the percent of  $^{125}\text{I}$ -erythropoietin bound. Counts bound by pre-immune sera were subtracted from all final

values to correct for nonspecific precipitation. The erythropoietin content of the unknown samples was determined by comparison to the standard curve.

The above procedure was applied to monkey serum obtained in Part A, above, as well as to the untreated monkey serum. Normal serum levels were assayed to contain approximately 36 mU/ml while treated monkey serum contained from 1000 to 1700 mU/ml.

5 Example 3

10 A. Monkey cDNA Library Construction

15 Messenger RNA was isolated from normal and anemia monkey kidneys by the guanidinium thiocyanate procedure of Chirgwin, et al., *Biochemistry*, 18, p. 5294 (1979) and poly (A)<sup>+</sup> mRNA was purified by two runs of oligo(dT)-cellulose column chromatography, as described at pp. 197-198 in Maniatis, et al., "Molecular Cloning, A Laboratory Manual" (Cold Springs Harbor Laboratory, Cold Springs, Harbor, N.Y., 1982). The cDNA library was constructed according to a modification of the general procedures of Okayama, et al., *Mol. and Cell. Biol.*, 2, pp. 161-170 (1982). The key features of the presently preferred procedures were as follows: (1) pUC8 was used as the sole vector, cut with *PstI* and then tailed with oligo dT of 60-80 bases in length; (2) *Hind* III digestion was used to remove the oligo dT tail from one end of the vector; (3) first strand synthesis and oligo dG tailing was carried out according to the published procedure; (4) *Bam* HI digestion was employed to remove the oligo dG tail from one end of the vector; and (5) replacement of the RNA strand by DNA was in the presence of two linkers

(GATCTAAAGACCGTCCCCCCCC and ACGGTCTTTA)

25 in a three-fold molar excess over the oligo dG tailed vector.

B. Colony Hybridization Procedures For Screening Monkey cDNA Library

30 Transformed *E. coli* were spread out at a density of 9000 colonies per 10 × 10 cm plate on nutrient plates containing 50 micrograms/ml Ampicillin. GeneScreen filters (New England Nuclear Catalog No. NEF-972) were pre-wet on a BHI-CAM plate (Bacto brain heart infusion 37 g/L, Casamino acids 2 g/L and agar 15 g/L, containing 500 micrograms/ml Chloramphenicol) and were used to lift the colonies off the plate. The colonies were grown in the same medium for 12 hours or longer to amplify the plasmid copy numbers. The amplified colonies (colony side up) were treated by serially placing the filters over 2 pieces of Whatman 3 MM paper saturated with each of the following solutions:

35 (1) 50 mM glucose—25 mM Tris-HCl (pH 8.0)—10 mM EDTA (pH 8.0) for five minutes;  
 (2) 0.5 M NaOH for ten minutes; and  
 (3) 1.0 M Tris-HCl (pH 7.5) for three minutes.

40 The filters were then air dried in a vacuum over at 80°C for two hours.

The filters were then subjected to Proteinase K digestion through treatment with a solution containing 50 micrograms/ml of the protease enzyme in Buffer K (0.1M Tris-HCl (pH 8.0)—0.15M NaCl—10 mM EDTA (pH 8.2)—0.2% SDS]. Specifically, 5 ml of the solution was added to each filter and the digestion was allowed to proceed at 55°C for 30 minutes, after which the solution was removed.

45 The filters were then treated with 4 ml of a prehybridization buffer (5 × SSPE—0.5% SDS—100 micrograms/ml SS *E. coli*/DNA—5 × BFP). The prehybridization treatment was carried out at 55°C, generally for 4 hours or longer, after which the prehybridization buffer was removed.

50 The hybridization process was carried out in the following manner. To each filter was added 3 ml of hybridization buffer (5 × SSPE—0.5% SDS—100 micrograms/ml yeast tRNA) containing 0.025 picomoles of each of the 128 probe sequences of Table II (the total mixture being designated the EPV mixture) and the filters were maintained at 48°C for 20 hours. This temperature was 2°C less than the lowest of the calculated dissociation temperatures (T<sub>d</sub>) determined for any of the probes.

55 Following hybridization, the filters were washed three times for ten minutes on a shaker with 6 × SSC—0.1% SDS at room temperature and washed two to three times with 6 × SSC-1% SDS at the hybridization temperature (48°C).

Autoradiography of the filters revealed seven positive clones among the 200,000 colonies screened.

Initial sequence analysis of one of the putative monkey cDNA clones (designated clone 83) was performed for verification purposes by a modification of the procedure of Wallace, et al., *Gene*, 16, pp. 21—26 (1981). Briefly, plasmid DNA from monkey cDNA clone 83 was linearized by digestion with *Eco* RI and denatured by heating in a boiling water

bath. The nucleotide sequence was determined by the dideoxy method of Sanger, et al., *P.N.A.S. (U.S.A.)* 74, pp. 5463—5467 (1977). A subset of the EPV mixture of probes consisting of 16 sequences was used as a primer for the sequencing reactions.

5 C. Monkey EPO cDNA Sequencing

Nucleotide sequence analysis of clone 83 was carried out by the procedures of Messing, *Methods in Enzymology*, 101, pp. 20—78 (1983). Set out in Table IV is a preliminary restriction map analysis of the approximately 1600 base pair *EcoRI/HindIII* cloned fragment of clone 83. Approximate locations of restriction endo-nuclease enzyme recognition sites are provided in terms of number of bases 3' to the *EcoRI* site at the 5' end of the fragment. Nucleotide sequencing was carried out by sequencing individual restriction fragments with the intent of matching overlapping fragments. For example, an overlap of sequence information provided by analysis of nucleotides in a restriction fragment designated C113 (*Sau3A* at ~111/*SmaI* at ~324) and the reverse order sequencing of a fragment designated C73 (*AluI* at ~424/*BstEII* at ~203).

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TABLE IV

Restriction Enzyme		
	Recognition Site	Approximate Location(s)
20	<u>EcoRI</u>	1
	<u>Sau3A</u>	111
	<u>SmaI</u>	180
10	<u>BstEII</u>	203
	<u>SmaI</u>	324
	<u>KpnI</u>	371
25	<u>RsaI</u>	372
	<u>AluI</u>	424
	<u>PstI</u>	426
30	<u>AluI</u>	430
	<u>HpaI</u>	466
	<u>AluI</u>	546
	<u>PstI</u>	601
35	<u>PvuII</u>	604
	<u>AluI</u>	605
	<u>AluI</u>	782
	<u>AluI</u>	788
40	<u>RsaI</u>	792
	<u>PstI</u>	807
	<u>AluI</u>	841
	<u>AluI</u>	927
	<u>NcoI</u>	946
45	<u>Sau3A</u>	1014
	<u>AluI</u>	1072
	<u>AluI</u>	1115
	<u>AluI</u>	1223
	<u>PstI</u>	1301
50	<u>RsaI</u>	1343
	<u>AluI</u>	1384
	<u>HindIII</u>	1449
	<u>AluI</u>	1450
55	<u>HindIII</u>	1585

Sequencing of approximately 1342 base pairs (within the region spanning the *Sau3A* site 3' to the *EcoRI* site and the *HindIII* site) and analysis of all possible reading frames has allowed for the development of DNA and amino acid

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sequence information set out in Table V. In the Table, the putative initial amino acid residue of the amino terminal of mature EPO (as verified by correlation to the previously mentioned sequence analysis of twenty amino terminal residues) is designated by the numeral +1. The presence of a methionine-specifying ATG codon (designated -27) "up-stream" of the initial amino terminal alanine residue as the first residue designated for the amino acid sequence of the mature protein is indicative of the likelihood that EPO is initially expressed in the cytoplasm in a precursor form including a 27 amino acid "leader" region which is excised prior to entry of mature EPO into circulation. Potential glycosylation sites within the polypeptide are designated by asterisks. The estimated molecular weight of the translated region was determined to be 21,117 daltons and the M.W. of the 165 residues of the polypeptide constituting mature monkey EPO was determined to be 18,236 daltons.

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TABLE V

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Translation of Monkey EPO cDNA

Sau3A

GATCCCCGGCCCCCTGGACAGCCGCCCTCTCCTCCAGGCCCCGTGGGGCTGGCCCTGCC  
 10 CGCTGAACCTCCCGGGATGAGGACTCCCGGTGTGGTCACCGCGCCCTAGGTGCGCTGAG

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-27 -20  
 Met Gly Val His Glu Cys Pro Ala Trp  
 GGACCCCCGGCCAGGCAGGGAGATG GGG GTG CAC GAA TGT CCT GCC TGG

20

-10  
 Leu Trp Leu Leu Leu Ser Leu Val Ser Leu Pro Leu Gly Leu Pro  
 CTG TGG CTT CTC CTG TCT CTC GTG TCG CTC CCT CTG GGC CTC CCA

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-1 +1 10  
 Val Pro Gly Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu  
 GTC CCG GGC GCC CCA CCA CGC CTC ATC TGT GAC AGC CGA GTC CTG

30

20 \* 40  
 Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Val Thr Met  
 GAG AGG TAC CTC TTG GAG GCC AAG GAG GCC GAG AAT GTC ACG ATG

35

30 \* 40  
 Gly Cys Ser Glu Ser Cys Ser Leu Asn Glu Asn Ile Thr Val Pro  
 GGC TGT TCC GAA AGC TGC AGC TTG AAT GAG AAT ATC ACC GTC CCA

45

50  
 Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly  
 GAC ACC AAA GTT AAC TTC TAT GCC TGG AAG AGG ATG GAG GTC GGG

40

60 70  
 Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu  
 CAG CAG GCT GTA GAA GTC TGG CAG GGC CTG GCC CTG CTC TCA GAA

80 \* 40  
 Ala Val Leu Arg Gly Gln Ala Val Leu Ala Asn Ser Ser Gln Pro  
 GCT GTC CTG CGG GGC CAG GCC GTG TTG GCC AAC TCT TCC CAG CCT

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90 100  
 Phe Glu Pro Leu Gln Leu His Met Asp Lys Ala Ile Ser Gly Leu  
 TTC GAG CCC CTG CAG CTG CAC ATG GAT AAA GCC ATC AGT GGC CTT

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110  
 Arg Ser Ile Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Glu Ala  
 CGC AGC ATC ACC ACT CTG CTT CGG GCG CTG GGA GCC CAG GAA GCC

TABLE V (continued)

5 120 130  
 Ile Ser Leu Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile  
 ATC TCC CTC CCA GAT GCG GCC TCG GCT GCT CCA CTC CGA ACC ATC  
 10 140  
 Thr Ala Asp Thr Phe Cys Lys Leu Phe Arg Val Tyr Ser Asn Phe  
 ACT GCT GAC ACT TTC TGC AAA CTC TTC CGA GTC TAC TCC AAT TTC  
 15 150 160  
 Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Arg  
 CTC CGG GGA AAG CTG AAG CTG TAC ACG GGG GAG GCC TGC AGG AGA  
 20 165  
 Gly Asp Arg OP  
 GGG GAC AGA TGA CCAGGTGCGTCCAGCTGGGCACATCCACCCACCTCCCTACCAACA  
 CTGCCTGTGCCACACCCCTCCCTCACCACTCCCGAACCCCATCGAGGGCTCTCAGCTAAC  
 25  
 CGCCAGCCTGTCCCATGGACACTCCAGTGCCAGCAATGACATCTCAGGGGCCAGAGGAAC  
 TGTCCAGAGCACAACCTTGAGATCTAAGGATGTCGAGGGCCAACCTGAGGGCCAGAGC  
 AGGAAGCATTCAAGAGAGCAGCTTAAACTCAGGAGCAGAGACAATGCAGGGAAAACACCT  
 30  
 GAGCTCACTCGGCCACCTGCAAAATTGATGCAGGACACGCTTGGAGGCAATTACCTG  
 TTTTGACCTACCATCAGGACAGGATGACTGGAGAACTTAGGTGGCAAGCTGTGACTT  
 CTCAAGGCCCTCACGGGCACTCCCTGGTGGCAAGAGCCCCCTTGACACTGAGAGAAATT  
 35  
 TTGCAATCTGCAGCAGGAAAATTACGGACAGGTTGGAGGTTGGAGGTTGGAGGGTACCTGACAG  
 GTGTGTCGGGAAGCAGGGCGTAGGGGTGGAGCTGGGATGCGAGTGAGAACCGTGAAGAC  
 40  
 AGGATGGGGCTGGCCTCTGGTTCTCGTGGGTCCAAGCTT  
 HindIII

45 The polypeptide sequence of Table V may readily be subjected to analysis for the presence of highly hydrophilic regions and/or secondary conformational characteristics indicative of potentially highly immunogenic regions by, e.g., the methods of Hopp, et al., *P.N.A.S. (U.S.A.)*, 78, pp. 3824-3828 (1981) and Kyte et al., *J.Mol.Biol.*, 157, pp. 105-132 (1982) and/or Chou, et al., *Biochem.*, 13, pp. 222-245 (1974) and *Advances in Enzymology*, 47, pp. 45-47 (1978). Computer-assisted analysis according to the Hopp, et al. method is available by means of a program designated PEP Reference Section 6.7 made available by Intelligenetics, Inc., 124 University Avenue, Palo Alto, California.

#### Example 4

#### A. Human Genomic Library

55 A Ch4A phage-borne human fetal liver genomic library prepared according to the procedures of Lawn, et al., *Cell*, 18, pp. 533-543 (1979) was obtained and maintained for use in a plaque hybridization assay.

B. Plaque Hybridization Procedures For Screening Human Genomic Library

5 Phage particles were lysed and the DNAs were fixed on filters (50,000 plaques per filter) according to the procedures of Woo, *Methods In Enzymology*, 68, pp. 389—395 (1979) except for the use of GeneScreen Plus filters (New England Nuclear Catalog No. NEF—976) and NZYAM plates (NaCl, 5 g; MgCl<sub>2</sub>—6H<sub>2</sub>O, 2 g; NZ-Amine A, 10 g; yeast extract, 5 g; casamino acids, 2 g; maltose, 2 g; and agar, 15 g per liter).

10 The air-dried filters were baked at 80°C for 1 hour and then digested with Proteinase K as described in Example 3, Part B. Prehybridization was carried out with a 1M NaCl—1% SDS buffer for 55°C for 4 hours or more, after which the buffer was removed. Hybridization and post-hybridization washings were carried out as described in Example 3, Part B. Both the mixture of 128 20-mer probes designated EPV and the mixture of 128 17-mer probes of Table III (designated the EPQ mixture) were employed. Hybridization was carried out at 48°C using the EPV probe mixture. EPQ probe mixture hybridization was carried out at 46°C—4 degrees below the lowest calculated T<sub>d</sub> for members of the mixture. Removal of the hybridized probe for rehybridization was accomplished by boiling with 1 X SSC — 0.1 % SDS for two minutes. Autoradiography of the filters revealed three positive clones (reactive with both probe mixtures) 15 among the 1,500,000 phage plaques screened. Verification of the positive clones as being EPO-encoding was obtained through DNA sequencing and electron micrographic visualization of heteroduplex formation with the monkey cDNA of Example 3. This procedure also gave evidence of multiple introns in the genomic DNA sequence.

20 Example 5

Nucleotide sequence analysis of one of the positive clones (designated AhEl) was carried out and results obtained to date are set out in Table VI.

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TABLE VI

5 AAGCTTCTGGCTTCCAGACCCAGCTACTTTCGGAAACTCAGCAACCCAGGCATCTGAGTCCTGGCCCA  
 10 AGACCGGGATGCCCTCAGGGAGGTGTCGGGAGCCCTGGCCAGCTTCCAGATAGCACGCTCGGCCAGTCCC  
 15 AAGGGTGGCAACCGGGTGCACTCCCTCCGGACCCAGGGGGAGGAGCCCCATGACCCACACGC  
 20 ACCTCTGCAGCAGCCCCGGCTCACGCCCGGGAGCCTCAACCCAGGCCTGCTGCCCTGCTCTGACCCGG  
 25 GTGGCCCTTACCCCTGGCCACCCCTACGGCACACAGCTCTCCCACCCCCAACCGGGCACGGCACATG  
 30 CAGATAACAGCCCCGAGCCCCGGCAGAGCGXAGAGTCCCCTGGCCACCCCCGGCCXGCTTGCTGCCCTG  
 35 CGCCGCACCGGGCTGTCCGGAGGCCAGGGGGCACCCGCCXGCTTGCTGCCACCCGGCCCC  
 40 CTTGGACAGCCGCCCTCTCCCTAGGCCGTGGGCTGGCCCTGGACCCGGAGCTTCCCCGGATGAGGXX  
 45 CCCGGTGACCGGGCCCAAGTCGCTGAGGGACCCGCCAAGGGGGAG  
 50 GAGTACTGGGGCTGGCCCTCCCCGGGGCTTCTGAGGGGGGATTAGGGCCCCGGCT

-27      -24  
 Met   Gly   Val   His  
 ATG   GGG   GTG   CAC   G

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TABLE VI (cont'd.)

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TABLE VI (cont'd.)

TGGTGGCCCCAACCATACCTGAAACTACGCCAAGGAGC<sup>\*</sup>AAAGCCAGCATCCTACCCCTGTGGCCAGGG  
 CCAGAGCCCTTCAGGACCCCTTGACTCCCCGGCTGTGTCATTTCAG  
 His Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr  
 CAC TGC AGC TTG AAT GAG AAT ATC ACT GTC ACC CCA GAC ACC AAA GTT AAT TTC TAT  
 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69 72 75 78 81 84 87 90 93 96 99 102 105 108 111 114 117 120 123 126 129 132 135 138 141 144 147 150 153 156 159 162 165 168 171 174 177 180 183 186 189 192 195 198 201 204 207 210 213 216 219 222 225 228 231 234 237 240 243 246 249 252 255 258 261 264 267 270 273 276 279 282 285 288 291 294 297 299 302 305 308 311 314 317 320 323 326 329 332 335 338 341 344 347 350 353 356 359 362 365 368 371 374 377 380 383 386 389 392 395 398 401 404 407 410 413 416 419 422 425 428 431 434 437 440 443 446 449 452 455 458 461 464 467 470 473 476 479 482 485 488 491 494 497 499 502 505 508 511 514 517 520 523 526 529 532 535 538 541 544 547 550 553 556 559 562 565 568 571 574 577 580 583 586 589 592 595 598 601 604 607 610 613 616 619 622 625 628 631 634 637 640 643 646 649 652 655 658 661 664 667 670 673 676 679 682 685 688 691 694 697 699 702 705 708 711 714 717 720 723 726 729 732 735 738 741 744 747 750 753 756 759 762 765 768 771 774 777 780 783 786 789 792 795 798 801 804 807 810 813 816 819 822 825 828 831 834 837 840 843 846 849 852 855 858 861 864 867 870 873 876 879 882 885 888 891 894 897 899 902 905 908 911 914 917 920 923 926 929 932 935 938 941 944 947 950 953 956 959 962 965 968 971 974 977 980 983 986 989 992 995 998 1001 1004 1007 1010 1013 1016 1019 1022 1025 1028 1031 1034 1037 1040 1043 1046 1049 1052 1055 1058 1061 1064 1067 1070 1073 1076 1079 1082 1085 1088 1091 1094 1097 1099 1102 1105 1108 1111 1114 1117 1120 1123 1126 1129 1132 1135 1138 1141 1144 1147 1150 1153 1156 1159 1162 1165 1168 1171 1174 1177 1180 1183 1186 1189 1192 1195 1198 1201 1204 1207 1210 1213 1216 1219 1222 1225 1228 1231 1234 1237 1240 1243 1246 1249 1252 1255 1258 1261 1264 1267 1270 1273 1276 1279 1282 1285 1288 1291 1294 1297 1299 1302 1305 1308 1311 1314 1317 1320 1323 1326 1329 1332 1335 1338 1341 1344 1347 1350 1353 1356 1359 1362 1365 1368 1371 1374 1377 1380 1383 1386 1389 1392 1395 1398 1401 1404 1407 1410 1413 1416 1419 1422 1425 1428 1431 1434 1437 1440 1443 1446 1449 1452 1455 1458 1461 1464 1467 1470 1473 1476 1479 1482 1485 1488 1491 1494 1497 1499 1502 1505 1508 1511 1514 1517 1520 1523 1526 1529 1532 1535 1538 1541 1544 1547 1550 1553 1556 1559 1562 1565 1568 1571 1574 1577 1580 1583 1586 1589 1592 1595 1598 1601 1604 1607 1610 1613 1616 1619 1622 1625 1628 1631 1634 1637 1640 1643 1646 1649 1652 1655 1658 1661 1664 1667 1670 1673 1676 1679 1682 1685 1688 1691 1694 1697 1699 1702 1705 1708 1711 1714 1717 1720 1723 1726 1729 1732 1735 1738 1741 1744 1747 1750 1753 1756 1759 1762 1765 1768 1771 1774 1777 1780 1783 1786 1789 1792 1795 1798 1801 1804 1807 1810 1813 1816 1819 1822 1825 1828 1831 1834 1837 1840 1843 1846 1849 1852 1855 1858 1861 1864 1867 1870 1873 1876 1879 1882 1885 1888 1891 1894 1897 1899 1902 1905 1908 1911 1914 1917 1920 1923 1926 1929 1932 1935 1938 1941 1944 1947 1950 1953 1956 1959 1962 1965 1968 1971 1974 1977 1980 1983 1986 1989 1992 1995 1998 2001 2004 2007 2010 2013 2016 2019 2022 2025 2028 2031 2034 2037 2040 2043 2046 2049 2052 2055 2058 2061 2064 2067 2070 2073 2076 2079 2082 2085 2088 2091 2094 2097 2099 2102 2105 2108 2111 2114 2117 2120 2123 2126 2129 2132 2135 2138 2141 2144 2147 2150 2153 2156 2159 2162 2165 2168 2171 2174 2177 2180 2183 2186 2189 2192 2195 2198 2201 2204 2207 2210 2213 2216 2219 2222 2225 2228 2231 2234 2237 2240 2243 2246 2249 2252 2255 2258 2261 2264 2267 2270 2273 2276 2279 2282 2285 2288 2291 2294 2297 2299 2302 2305 2308 2311 2314 2317 2320 2323 2326 2329 2332 2335 2338 2341 2344 2347 2350 2353 2356 2359 2362 2365 2368 2371 2374 2377 2380 2383 2386 2389 2392 2395 2398 2401 2404 2407 2410 2413 2416 2419 2422 2425 2428 2431 2434 2437 2440 2443 2446 2449 2452 2455 2458 2461 2464 2467 2470 2473 2476 2479 2482 2485 2488 2491 2494 2497 2499 2502 2505 2508 2511 2514 2517 2520 2523 2526 2529 2532 2535 2538 2541 2544 2547 2550 2553 2556 2559 2562 2565 2568 2571 2574 2577 2580 2583 2586 2589 2592 2595 2598 2601 2604 2607 2610 2613 2616 2619 2622 2625 2628 2631 2634 2637 2640 2643 2646 2649 2652 2655 2658 2661 2664 2667 2670 2673 2676 2679 2682 2685 2688 2691 2694 2697 2699 2702 2705 2708 2711 2714 2717 2720 2723 2726 2729 2732 2735 2738 2741 2744 2747 2750 2753 2756 2759 2762 2765 2768 2771 2774 2777 2780 2783 2786 2789 2792 2795 2798 2801 2804 2807 2810 2813 2816 2819 2822 2825 2828 2831 2834 2837 2840 2843 2846 2849 2852 2855 2858 2861 2864 2867 2870 2873 2876 2879 2882 2885 2888 2891 2894 2897 2899 2902 2905 2908 2911 2914 2917 2920 2923 2926 2929 2932 2935 2938 2941 2944 2947 2950 2953 2956 2959 2962 2965 2968 2971 2974 2977 2980 2983 2986 2989 2992 2995 2998 3001 3004 3007 3010 3013 3016 3019 3022 3025 3028 3031 3034 3037 3040 3043 3046 3049 3052 3055 3058 3061 3064 3067 3070 3073 3076 3079 3082 3085 3088 3091 3094 3097 3099 3102 3105 3108 3111 3114 3117 3120 3123 3126 3129 3132 3135 3138 3141 3144 3147 3150 3153 3156 3159 3162 3165 3168 3171 3174 3177 3180 3183 3186 3189 3192 3195 3198 3201 3204 3207 3210 3213 3216 3219 3222 3225 3228 3231 3234 3237 3240 3243 3246 3249 3252 3255 3258 3261 3264 3267 3270 3273 3276 3279 3282 3285 3288 3291 3294 3297 3299 3302 3305 3308 3311 3314 3317 3320 3323 3326 3329 3332 3335 3338 3341 3344 3347 3350 3353 3356 3359 3362 3365 3368 3371 3374 3377 3380 3383 3386 3389 3392 3395 3398 3401 3404 3407 3410 3413 3416 3419 3422 3425 3428 3431 3434 3437 3440 3443 3446 3449 3452 3455 3458 3461 3464 3467 3470 3473 3476 3479 3482 3485 3488 3491 3494 3497 3499 3502 3505 3508 3511 3514 3517 3520 3523 3526 3529 3532 3535 3538 3541 3544 3547 3550 3553 3556 3559 3562 3565 3568 3571 3574 3577 3580 3583 3586 3589 3592 3595 3598 3601 3604 3607 3610 3613 3616 3619 3622 3625 3628 3631 3634 3637 3640 3643 3646 3649 3652 3655 3658 3661 3664 3667 3670 3673 3676 3679 3682 3685 3688 3691 3694 3697 3699 3702 3705 3708 3711 3714 3717 3720 3723 3726 3729 3732 3735 3738 3741 3744 3747 3750 3753 3756 3759 3762 3765 3768 3771 3774 3777 3780 3783 3786 3789 3792 3795 3798 3801 3804 3807 3810 3813 3816 3819 3822 3825 3828 3831 3834 3837 3840 3843 3846 3849 3852 3855 3858 3861 3864 3867 3870 3873 3876 3879 3882 3885 3888 3891 3894 3897 3899 3902 3905 3908 3911 3914 3917 3920 3923 3926 3929 3932 3935 3938 3941 3944 3947 3950 3953 3956 3959 3962 3965 3968 3971 3974 3977 3980 3983 3986 3989 3992 3995 3998 4001 4004 4007 4010 4013 4016 4019 4022 4025 4028 4031 4034 4037 4040 4043 4046 4049 4052 4055 4058 4061 4064 4067 4070 4073 4076 4079 4082 4085 4088 4091 4094 4097 4099 4102 4105 4108 4111 4114 4117 4120 4123 4126 4129 4132 4135 4138 4141 4144 4147 4150 4153 4156 4159 4162 4165 4168 4171 4174 4177 4180 4183 4186 4189 4192 4195 4198 4201 4204 4207 4210 4213 4216 4219 4222 4225 4228 4231 4234 4237 4240 4243 4246 4249 4252 4255 4258 4261 4264 4267 4270 4273 4276 4279 4282 4285 4288 4291 4294 4297 4299 4302 4305 4308 4311 4314 4317 4320 4323 4326 4329 4332 4335 4338 4341 4344 4347 4350 4353 4356 4359 4362 4365 4368 4371 4374 4377 4380 4383 4386 4389 4392 4395 4398 4401 4404 4407 4410 4413 4416 4419 4422 4425 4428 4431 4434 4437 4440 4443 4446 4449 4452 4455 4458 4461 4464 4467 4470 4473 4476 4479 4482 4485 4488 4491 4494 4497 4499 4502 4505 4508 4511 4514 4517 4520 4523 4526 4529 4532 4535 4538 4541 4544 4547 4550 4553 4556 4559 4562 4565 4568 4571 4574 4577 4580 4583 4586 4589 4592 4595 4598 4601 4604 4607 4610 4613 4616 4619 4622 4625 4628 4631 4634 4637 4640 4643 4646 4649 4652 4655 4658 4661 4664 4667 4670 4673 4676 4679 4682 4685 4688 4691 4694 4697 4699 4702 4705 4708 4711 4714 4717 4720 4723 4726 4729 4732 4735 4738 4741 4744 4747 4750 4753 4756 4759 4762 4765 4768 4771 4774 4777 4780 4783 4786 4789 4792 4795 4798 4801 4804 4807 4810 4813 4816 4819 4822 4825 4828 4831 4834 4837 4840 4843 4846 4849 4852 4855 4858 4861 4864 4867 4870 4873 4876 4879 4882 4885 4888 4891 4894 4897 4899 4902 4905 4908 4911 4914 4917 4920 4923 4926 4929 4932 4935 4938 4941 4944 4947 4950 4953 4956 4959 4962 4965 4968 4971 4974 4977 4980 4983 4986 4989 4992 4995 4998 5001 5004 5007 5010 5013 5016 5019 5022 5025 5028 5031 5034 5037 5040 5043 5046 5049 5052 5055 5058 5061 5064 5067 5070 5073 5076 5079 5082 5085 5088 5091 5094 5097 5099 5102 5105 5108 5111 5114 5117 5120 5123 5126 5129 5132 5135 5138 5141 5144 5147 5150 5153 5156 5159 5162 5165 5168 5171 5174 5177 5180 5183 5186 5189 5192 5195 5198 5201 5204 5207 5210 5213 5216 5219 5222 5225 5228 5231 5234 5237 5240 5243 5246 5249 5252 5255 5258 5261 5264 5267 5270 5273 5276 5279 5282 5285 5288 5291 5294 5297 5299 5302 5305 5308 5311 5314 5317 5320 5323 5326 5329 5332 5335 5338 5341 5344 5347 5350 5353 5356 5359 5362 5365 5368 5371 5374 5377 5380 5383 5386 5389 5392 5395 5398 5401 5404 5407 5410 5413 5416 5419 5422 5425 5428 5431 5434 5437 5440 5443 5446 5449 5452 5455 5458 5461 5464 5467 5470 5473 5476 5479 5482 5485 5488 5491 5494 5497 5499 5502 5505 5508 5511 5514 5517 5520 5523 5526 5529 5532 5535 5538 5541 5544 5547 5550 5553 5556 5559 5562 5565 5568 5571 5574 5577 5580 5583 5586 5589 5592 5595 5598 5601 5604 5607 5610 5613 5616 5619 5622 5625 5628 5631 5634 5637 5640 5643 5646 5649 5652 5655 5658 5661 5664 5667 5670 5673 5676 5679 5682 5685 5688 5691 5694 5697 5699 5702 5705 5708 5711 5714 5717 5720 5723 5726 5729 5732 5735 5738 5741 5744 5747 5750 5753 5756 5759 5762 5765 5768 5771 5774 5777 5780 5783 5786 5789 5792 5795 5798 5801 5804 5807 5810 5813 5816 5819 5822 5825 5828 5831 5834 5837 5840 5843 5846 5849 5852 5855 5858 5861 5864 5867 5870 5873 5876 5879 5882 5885 5888 5891 5894 5897 5899 5902 5905 5908 5911 5914 5917 5920 5923 5926 5929 5932 5935 5938 5941 5944 5947 5950 5953 5956 5959 5962 5965 5968 5971 5974 5977 5980 5983 5986 5989 5992 5995 5998 6001 6004 6007 6010 6013 6016 6019 6022 6025 6028 6031 6034 6037 6040 6043 6046 6049 6052 6055 6058 6061 6064 6067 6070 6073 6076 6079 6082 6085 6088 6091 6094 6097 6099 6102 6105 6108 6111 6114 6117 6120 6123 6126 6129 6132 6135 6138 6141 6144 6147 6150 6153 6156 6159 6162 6165 6168 6171 6174 6177 6180 6183 6186 6189 6192 6195 6198 6201 6204 6207 6210 6213 6216 6219 6222 6225 6228 6231 6234 6237 6240 6243 6246 6249 6252 6255 6258 6261 6264 6267 6270 6273 6276 6279 6282 6285 6288 6291 6294 6297 6299 6302 6305 6308 6311 6314 6317 6320 6323 6326 6329 6332 6335 6338 6341 6344 6347 6350 6353 6356 6359 6362 6365 6368 6371 6374 6377 6380 6383 6386 6389 6392 6395 6398 6401 6404 6407 6410 6413 6416 6419 6422 6425 6428 6431 6434 6437 6440 6443 6446 6449 6452 6455 6458 6461 6464 6467 6470 6473 6476 6479 6482 6485 6488 6491 6494 6497 6499 6502 6505 6508 6511 6514 6517 6520 6523 6526 6529 6532 6535 6538 6541 6544 6547 6550 6553 6556 6559 6562 6565 6568 6571 6574 6577 6580 6583 6586 6589 6592 6595 6598 6601 6604 6607 6610 6613 6616 6619 6622 6625 6628 6631 6634 6637 6640 6643 6646 6649 6652 6655 6658 6661 6664 6667 6670 6673 6676 6679 6682 6685 6688 6691 6694 6697 6699 6702 6705 6708 6711 6714 6717 6720 6723 6726 6729 6732 6735 6738 6741 6744 6747 6750 6753 6756 6759 6762 6765 6768 6771 6774 6777 6780 6783 6786 6789 6792 6795 6798 6801 6804 6807 6810 6813 6816 6819 6822 6825 6828 6831 6834 6837 6840 6843 6846 6849 6852 6855 6858 6861 6864 6867 6870 6873 6876 6879 6882 6885 6888 6891 6894 6897 6899 6902 6905 6908 6911 6914 6917 6920 6923 6926 6929 6932 6935 6938 6941 6944 6947 6950 6953 6956 6959 6962 6965 6968 6971 6974 6977 6980 6983 6986 6989 6992 6995 6998 7001 7004 7007 7010 7013 7016 7019 7022 7025 7028 7031 7034 7037 7040 7043 7046 7049 7052 7055 7058 7061 7064 7067 7070 7073 7076 7079 7082 7085 7088 7091 7094 7097 7099 7102 7105 7108 7111 7114 7117 7120 7123 7126 7129 7132 7135 7138 7141 7144 7147 7150 7153 7156 7159 7162 7165 7168 7171 7174 7177 7180 7183 7186 7189 7192 7195 7198 7201 7204 7207 7210 7213 7216 7219 7222 7225 7228 7231 7234 7237 7240 7243 7246 7249 7252 7255 7258 7261 7264 7267 7270 7273 7276 7279 7282 7285 7288 7291 7294 7297 7299 7302 7305 7308 7311 7314 7317 7320 7323 7326 7329 7332 7335 7338 7341 7344 7347 7350 7353 7356 7359 7362 7365 7368 7371 7374 7377 7380 7383 7386 7389 7392 7395 7398 7401 7404 7407 7410 7413 7416 7419 7422 7425 7428 7431 7434 7437 7440 7443 7446 7449 7452 7455 7458 7461 7464 7467 7470 7473 7476 7479 7482 7485 7488 7491 7494 7497 7499 7502 7505 7508 7511 7514 7517 7520 7523 7526 7529 7

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TABLE VI (cont'd.)

AAAAGAGAAAAGAAAATATGAGGGCTGATGGAAATACATTCACTCACTCACT	
CACTCATTCACTCATTCATTCAACAAAGCTTATGGATACCTTCGCTAGCTGGCTGG	
GGCTGCTGAGGGCAGGAGGGTACATGGCTAGCTCAGAGTCCACTCCCTGTAG	
56 Val GLY Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala	60
GTC GGG CAG CAG CCC GTC GAA GTC GTC TGG CAG EGC CTG GCC CTG TCG TCG GAA GCT	
70 Val Leu Arg GLY Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Itp Glu Pro Leu	
GTC CTG CGG CCC CAG GGC CAG GTC AAC TCT TCC CAG CCC TGG GAG CCC CTG	
80 * Val Leu Arg GLY Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Itp Glu Pro Leu	
CAG CTG CAT GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG CTT	
90 Gln Leu His Val Asp Lys Ala Val Ser Gln Leu Arg Ser Leu Thr Thr Leu Leu	
CAG CTG GCT GGA GGC CAG GTGAGTAGGAGGGACACTTCGCTTGGCCCTTCTGTAAGAAGGGAA	
100 GAGGGCTTGGCTAAGGAGTACAGGAACGTGCCGTATTCCTTCCCTTCTGAGGACTGAGCCTCC	
110 Arg Ala Leu Gly Ala Gln CAG GCT CTG GGA GGC CAG	115
116 LYS AAG GAA GGC ATC TCC CCT CCA GAT GCC GCG TCA GCT GCT	120 Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala

TABLE VI (cont'd.)

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser  
 CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC CGC AAA CTC TTC CGA GTC TAC TCC 140  
 Asn Phe Leu Arg Gly Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly  
 AAT TTC CTC CGG GGA AAG CTG AAG CTG TAC ACA GGG GAG GCC TCC AGG ACA GGG 150  
 160  
 ASP ARG OP  
 GAC AGA TGA CCAGGTGTGCCACCTGGCATATCCACCACTCCCTCACCAACATTGCTTGTGCCACA  
 CCCCCCCGGCACTCCCTGAACCCCGTCAAGGGCCAGAGGAACACTGTCAGCTAGGCCAGGCTGTCCCATGGACACTCC  
 AGTGCCAGCAATGACATCTCAAGGGCCAGAGGAACACTGTCAGAGGAAGCATTCAAGAGGCCATTAAACTCAGGGACAGGCCATGC  
 AGGGCCAACTTGAGGGCCCAGAGCAGGAAGCATTCAAGAGGCCATTAAACTCAGGGACAGGCCATTAC  
 TGGGAAGACGGCTGAGCTCACCTGGCACCCCTGCAAAATTGATGCCAGGACACGGCTTGGAGGGATTAC  
 CTGTTTGGCACCTACCATCAAGGGACAGGATGACCTGGAGAACATTAGTGGCAAGCTGAGCTTCTCCAGG  
 TCTCACGGGCATGGGCACTCCCTGGCAAGAGCCCCCTTGACACCGGGTGGTGGAACCATGAAAGAC  
 AXGATXGGGGCTGGCCTCTGGCTCACACTGGGTCACAGCTTGTATTCTACACCTATTGACAGACTGAA  
 ACACAAATATGAC

45 In Table VI, the initial continuous DNA sequence designates a top strand of 620 bases in what is apparently an untranslated sequence immediately preceding a translated portion of the human EPO gene. More specifically, the sequence appears to comprise the 5' end of the gene which leads up to a translated DNA region coding for the first four amino acids (-27 through -24) of a leader sequence ("presequence"). Four base pairs in the sequence prior to that encoding the beginning of the leader have not yet been unambiguously determined and are therefore designated by an "X". There then follows an intron of about 639 base pairs (439 base pairs of which have been sequenced and the remaining 200 base pairs of which are designated "I.S.") and immediately preceding a codon for glutamine which has been designated as residue -23 of the translated polypeptide. The exon sequence immediately following is seen to code for amino acid residues through an alanine residue (designated as the +1 residue of the amino acid sequence of mature human EPO) to the codon specifying threonine at position +26, whereupon there follows a second intron consisting of 256 bases as specifically designated. Following this intron is an exon sequence for amino acid residues 27 through 55 and thereafter a third intron comprising 612 base pairs commences. The subsequent exon codes for residues 56 through 115 of human EPO and there then commences a fourth intron of 134 bases as specified. Following the fourth intron is an exon coding for residue Nos. 116 through 166 and a "stop" codon (TGA). Finally, Table VI identifies

a sequence of 568 base pairs in what appears to be an untranslated 3' region of the human EPO gene, two base pairs of which ("X") have not yet been unambiguously sequenced.

5 Table VI thus serves to identify the primary structural conformation (amino acid sequence) of mature human EPO as including 166 specified amino acid residues (estimated M.W. = 18,399). Also revealed in the Table is the DNA sequence coding for a 27 residue leader sequence along with 5' and 3' DNA sequences which may be significant to promoter/operator functions of the human gene operon. Sites for potential glycosylation of the mature human EPO polypeptide are designated in the Table by asterisks. It is worthy of note that the specific amino acid sequence of Table VI likely constitutes that of a naturally occurring allelic form of human erythropoietin. Support for this position is found in the results of continued efforts at sequencing of urinary isolates of human erythropoietin which provided the finding 10 that a significant number of erythropoietin molecules therein have a methionine at residue 126 as opposed to a serine as shown in the Table.

15 Table VII, below, illustrates the extent of polypeptide sequence homology between human and monkey EPO. In the upper continuous line of the Table, single letter designations are employed to represent the deduced translated polypeptide sequences of human EPO commencing with residue -27 and the lower continuous line shows the deduced 20 polypeptide sequence of monkey EPO commencing at assigned residue number -27. Asterisks are employed to highlight the sequence homologies. It should be noted that the deduced human and monkey EPO sequences reveal an "additional" lysine (K) residue at (human) position 116. Cross-reference to Table VI indicates that this residue is at the margin of a putative mRNA splice junction in the genomic sequence. Presence of the lysine residue in the human polypeptide sequence was further verified by sequencing of a cDNA human sequence clone prepared from mRNA isolated from COSP—1 cells transfected with the human genomic DNA in Example 7, *infra*.

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## Example 6

The expression system selected for initial attempts at microbial synthesis of isolatable quantities of EPO polypeptide material coded for by the monkey cDNA provided by the procedures of Example 3 was one involving mammalian host cells (i.e., COS-1 cells, A.T.C.C. No. CRL-1650). The cells were transfected with a "shuttle" vector capable of autonomous replication in *E. coli* host (by virtue of the presence of pBR322-derived DNA) and the mammalian hosts (by virtue of the presence of SV40 virus-derived DNA).

More specifically, an expression vector was constructed according to the following procedures. The plasmid clone

TABLE VII  
Comparison of Human and Monkey EPO Polypeptides

	Human	Monkey	Human	Monkey	Human	Monkey	Human	Monkey
	-20	-10			20		30	
	MGVHECPAWLWLLSLPLGLPVLGAPRLLICDSRVLERYLLEAKEAENITIGCAEHCSLNENITVPDTK	*****					40	
	*****	*****						
	MGVHECPAWLWLLSLVSLPLGLPVLGAPRLLICDSRVLERYLLEAKEAENVTMGCSECSCLNENITVPDTK	*****						
	50	60	70		80		90	
	VNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQQPWEPLQLHVOKAVSGLRSLTTLRLALGQKE	*****	*****		*****		100	
	*****	*****	*****		*****		110	
	VNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQAVLANSQQPFEPLQLHMDKAISGLRSITTLRLALGQ-E	*****	*****		*****			
	120	130	140		150		160	
	AISPPDAASAAAPLRTITADTFRKLFRVYSNFLRGKLLYTGAEACRTGDR	*****	*****		*****			
	*****	*****	*****		*****			
	AISLPDAASAAAPLRTITADTFCKLFRVYSNFLRGKLLYTGAEACRRGDR	*****	*****		*****			

83 provided in Example 3 was amplified in *E.coli* and the approximately 1.4 kb monkey EPO-encoding DNA was isolated by EcoRI and *Hind*III digestion. Separately isolated was an approximately 4.0 kb, *Hind*III/*Sal*I fragment from pBR322. An approximately 30 bp, EcoRI/*Sal*I "linker" fragment was obtained from M13mp10 RF DNA (P and L Laboratories). This linker included, in series, an EcoRI sticky end, followed by *Sst*I, *Smal*, *Bam*HI and *Xba*I recognition sites and a

5 *Sal*I sticky end. The above three fragments were ligated to provide an approximately 5.4 kb intermediate plasmid ("pERS") wherein the EPO DNA was flanked on one side by a "bank" of useful restriction endonuclease recognition sites. pERS was then digested with *Hind*III and *Sal*I to yield the EPO DNA and the EcoRI to *Sal*I (M13mp10) linker. The 1.4 kb fragment was ligated with an approximately 4.0 kb *Bam*HI/*Sal*I of pBR322 and another M13mp10 *Hind*III/*Bam*HI RF fragment linker also having approximately 30 bp. The M13 linker fragment was characterized by a *Hind*III sticky end, followed by *Pst*I, *Sal*I, *Xba*I recognition sites and a *Bam*HI sticky end. The ligation product was, again, a useful intermediate plasmid ("pBR-EPO") including the EPO DNA flanked on both sides by banks of restriction site.

10 The vector chosen for expression of the EPO DNA in COS-1 cells ("pDSVL1") had previously been constructed to allow for selection and autonomous replication in *E.coli*. These characteristics are provided by the origin of replication and Ampicillin resistance gene DNA sequences present in the region spanning nucleotides 2448 through 4362 of pBR322. This sequence was structurally modified by the addition of a linker providing a *Hind*III recognition immediately adjacent nucleotide 2448 prior to incorporation into the vector. Among the selected vector's other useful properties was the capacity to autonomously replicate in COS-1 cells and the presence of a viral promoter sequence functional in mammalian cells. These characteristics are provided by the origin of replication DNA sequence and "late gene" viral promoter DNA sequence present in the 342 bp sequence spanning nucleotide numbers 5171 through 270 of the SV40 genome. A unique restriction site (*Bam*HI) was provided in the vector and immediately adjacent the viral promoter sequence through use of a commercially available linker sequence (Collaborative Research). Also incorporated in the vector was a 237 base pair sequence (derived as nucleotide numbers 2553 through 2770 of SV40) containing the "late gene" viral mRNA polyadenylation signal (commonly referred to as a transcription terminator). This fragment was positioned in the vector in the proper orientation *vis-a-vis* the "late gene" viral promoter via the unique *Bam*HI site. Also 15 present in the vector was another mammalian gene at a location not material to potential transcription of a gene inserted at the unique *Bam*HI site, between the viral promoter and terminator sequences. [The mammalian gene comprised an approximately 2,500 bp mouse dihydrofolate reductase (DHFR) minigene isolated from plasmid pMG-1 as in Gasser, et al., *P.N.A.S. (U.S.A.)*, 79, pp. 6522-6526, (1982).] Again, the major operative components of plasmid pDSVL1 comprise nucleotides 2448 through 4362 of pBR322 along with nucleotides 5171 through 270 (342 bp) and 2553 through 2770 (237 bp) of SV40 DNA.

20 Following procedures described, e.g., in Maniatis, et al., *supra*, the EPO-encoding DNA was isolated from plasmid pBR-EPO as a *Bam*HI fragment and ligated into plasmid pDSVL1 cut with *Bam*HI. Restriction enzyme analysis was employed to confirm insertion of the EPO gene in the correct orientation in two of the resulting cloned vectors (duplicate vectors H and L). See Figure 2, illustrating plasmid pDSVL-MkE. Vectors with EPO genes in the wrong orientation 25 were saved for use as negative controls in transfection experiments designed to determine EPO expression levels in hosts transformed with vectors having EPO DNA in the correct orientation.

25 Vectors H, L, F, X and G were combined with carrier DNA (mouse liver and spleen DNA) were employed to transfect duplicate 60 mm plates by calcium phosphate microprecipitate methods. Duplicate 60 mm plates were also transfected with carrier DNA as a "mock" transformation negative control. After five days all culture media were tested for the 30 presence of polypeptides possessing the immunological properties of naturally-occurring EPO.

#### Example 7

##### A. Initial EPO Expression System Involving COS-1 Cells

35 The system selected for initial attempts at microbial synthesis of isolatable quantities of human EPO polypeptide material coded for by the human genomic DNA EPO clone, also involved expression in mammalian host cells (i.e., COS-1 cells, A.T.C.C. No. CRL-1650). The human EPO gene was first sub-cloned into a "shuttle" vector which is capable of autonomous replication in both *E.coli* hosts (by virtue of the presence of pBR322 derived DNA) and in the 40 mammalian cell line COS-1 (by virtue of the presence of SV40 virus derived DNA). The shuttle vector, containing the EPO gene, was then transfected into COS-1 cells. EPO polypeptide material was produced in the transfected cells and secreted into the cell culture media.

45 More specifically, an expression vector was constructed according to the following procedures. DNA isolated from lambda clone  $\lambda$ hE1, containing the human genomic EPO gene, was digested with *Bam*HI and *Hind*III restriction endonucleases, and a 5.6 Kb DNA fragment known to contain the entire EPO gene was isolated. This fragment was mixed and ligated with the bacterial plasmid pUC8 (Bethesda Research Laboratories, Inc.) which had been similarly digested, creating the intermediate plasmid "pUC8-HuE", providing a convenient source of this restriction fragment.

50 The vector chosen for expression of the EPO DNA in COS-1 cells (pSV4SE1) had previously been constructed.

5 Plasmid pSV4SEt contained DNA sequences allowing selection and autonomous replication in *E.coli*. These characteristics are provided by the origin of replication and Ampicillin resistance gene DNA sequences present in the region spanning nucleotides 2448 through 4362 of the bacterial plasmid pBR322. This sequence was structurally modified by the addition of a linker providing a *Hind*III recognition site immediately adjacent to nucleotide 2448. Plasmid pSV4SEt was also capable of autonomous replication in COS—1 cells. This characteristic was provided by a 342 bp fragment containing the the SV40 virus origin of replication (nucleotide numbers 5171 through 270). This fragment has been modified by the addition of a linker providing an *Eco*RI recognition site adjacent to nucleotide 270 and a linker providing a *Sa*lI recognition site adjacent nucleotide 5171. A 1061 bp fragment of SV40 was also present in this vector (nucleotide numbers 1711 through 2772 plus a linker providing a *Sa*lI recognition site next to nucleotide number 2772). Within this fragment was an unique *Bam*HI recognition sequence. In summary, plasmid pSV4SEt contained unique *Bam*HI and *Hind*III recognition sites, allowing insertion of the human EPO gene, sequences allowing replication and selection in *E.coli*, and sequences allowing replication in COS—1 cells.

10 15 In order to insert the EPO gene into pSV4SEt, plasmid pUC8—HuE was digested with *Bam*HI and *Hind*III restriction endonucleases and the 5.6 kb EPO encoding DNA fragment isolated. pSV4SEt was also digested with *Bam*HI and *Hind*III and the major 2513 bp fragment isolated (preserving all necessary functions). These fragments were mixed and ligated, creating the final vector "pSVgHuEPO". (See, Figure 3.) This vector was propagated in *E.coli* and vector DNA isolated. Restriction enzyme analysis was employed to confirm insertion of the EPO gene.

20 25 Plasmid pSVgHuEPO DNA was used to express human EPO polypeptide material in COS—1 cells. More specifically, pSVgHuEPO DNA was combined with carrier DNA and transfected into triplicate 60 mm plates of COSP-1 cells. As a control, carrier DNA alone was also transfected into COS—1 cells. Cell culture media were sampled five and seven days later and tested for the presence of polypeptides possessing the immunological properties of naturally occurring human EPO.

#### B. Second EPO Expression System Involving COS—1 Cells

25 30 Still another system was designed to provide improved production of human EPO polypeptide material coded by the human genomic DNA EPO clone in COS—1 cells (A.T.C.C. No. CRL—1650).

35 In the immediately preceding system, EPO was expressed in COS—1 cells using its own promoter which is within the 5.6 Kb *Bam*HI to *Hind*III restriction fragment. In the following construction, the EPO gene is altered to that it is expressed using the SV40 late promoter.

40 45 More specifically, the cloned 5.6 Kb *Bam*HI to *Hind*III genomic human EPO restriction fragment was modified by the following procedures. Plasmid pUC8—HuE, as described above, was cleaved with *Bam*HI and with *Bst*EEI restriction endonucleases. *Bst*EEI cleaves within the 5.6 Kb EPO gene at a position which is 44 base pairs 5' to the initiating ATG coding for the pre-peptide and approximately 680 base pairs 3' to the *Hind*III restriction site. The approximately 4900 base pair fragment was isolated. A synthetic linker DNA fragment, containing *Sa*lI and *Bst*EEI sticky ends and an internal *Bam*HI recognition site was synthesized and purified. The two fragments were mixed and ligated with plasmid pBR322 which had been cut with *Sa*lI and *Bam*HI to produce the intermediate plasmid pBRgHE. The genomic human EPO gene can be isolated therefrom as a 4900 base pair *Bam*HI digestion fragment carrying the complete structural gene with a single ATG 44 base pairs 3' to *Bam*HI site adjacent the amino terminal coding region.

40 45 This fragment was isolated and inserted as a *Bam*HI fragment into *Bam*HI cleaved expression vector plasmid pDSVL1 (described in Example 6). The resulting plasmid, pSLVgHuEPO, as illustrated in Figure 4, was used to express EPO polypeptide material from COS—1 cells, as described in Examples 6 and 7A.

#### Example 8

45 50 Culture media from growth of the six transfected COS—1 cultures of Example 6 were analyzed by radioimmunoassay according to the procedures set forth in Example 2, Part B. Each sample was assayed at 250, 125, 50, and 25 microliter aliquot levels. Supernatants from growth of cells mock transfected or transfected with vectors having incorrect EPO gene orientation were unambiguously negative for EPO immunoreactivity. For each sample of the two supernatants derived from growth of COS—1 cells transfected with vectors (H and L) having the EPO DNA in the correct orientation, the % inhibition of  $^{125}$ I—EPO binding to antibody ranged from 72 to 88%, which places all values at the top of the standard curve. The exact concentration of EPO in the culture supernatant could not then reliably be estimated. A quite conservative estimate of 300 mU/ml was made, however, from the value calculation of the largest aliquot size (250 microliter).

55 60 A representative culture fluid according to Example 6 and five and seven day culture fluids obtained according to Example 7A were tested in the RIA in order to compare activity of recombinant monkey and human EPO materials to a naturally-occurring human EPO standard and the results are set out in graphic form in Figure 1. Briefly, the results expectedly revealed that the recombinant monkey EPO significantly competed for anti-human EPO antibody although

5 it was not able to completely inhibit binding under the test conditions. The maximum percent inhibition values for recombinant human EPO, however, closely approximated those of the human EPO standard. The parallel nature of the dose response curves suggest immunological identity of the sequences (epitopes) in common. Prior estimates of monkey EPO in culture fluids were re-evaluated at these higher dilution levels and were found to range from 2.91 to 3.12 U/ml. Estimated human EPO production levels were correspondingly set at 302 mU/ml for the five-day growth sample and 567 mU/ml for the seven day growth sample. Estimated monkey EPO production levels in the Example 7B expression system were on the same order or better.

## 10 Example 9

15 Culture fluids prepared according to Examples 6 and 7 were subjected to an *in vitro* assay for EPO activity according to the procedure of Goldwasser, et al., *Endocrinology*, 97, 2, pp. 315—323 (1975). Estimated monkey EPO values for culture fluids tested ranged from 3.2 to 4.3 U/ml. Human EPO culture fluids were also active in this *in vitro* assay and, further, this activity could be neutralized by anti-EPO antibody. The recombinant monkey EPO culture fluids according to Example 6 were also subjected to an assay for *in vivo* biological activity according to the general procedures of Cotes, et al., *Nature*, 191, pp. 1065—1067 (1961) and Hammond, et al., *Ann. N. Y. Acad. Sci.*, 149, pp. 516—527 (1968) and activity levels ranged from 0.94 to 1.24 U/ml.

## 20 Example 10

25 In the previous examples, recombinant monkey or human EPO material was produced from vectors used to transfect COS—1 cells. These vectors replicate in COS—1 cells due to the presence of SV40 T antigen within the cell and an SV40 origin of replication on the vectors. Though these vectors produce useful quantities of EPO in COS—1 cells, expression is only transient (7 to 14 days) due to the eventual loss of the vector. Additionally, only a small percentage of COS—1 became productively transfected with the vectors. The present example describes expression systems employing Chinese hamster ovary (CHO) DHFR- cells and the selectable marker, DHFR. [For discussion of related expression systems, see U.S. Letters Patent No. 4,399,216 and European Patent Applications 117058, 117059 and 117060, all published August 29, 1984.]

30 CHO DHFR- cells (DuX—B11) CHD K1 cells, Urlaub, et al., *Proc. Nat. Acad. Sci. (U.S.A.)*, Vol. 77, 4461 (1980) lack the enzyme dihydrofolate reductase (DHFR) due to mutations in the structural genes and therefore require the presence of glycine, hypoxanthine, and thymidine in the culture media. Plasmids pDSVL-MkE (Example 6) or pDSVL-gHuEPO (Example 7B) were transfected along with carrier DNA into CHO DHFR- cells growing in media containing hypoxanthine, thymidine, and glycine in 60 mm culture plates. Plasmid pSVgHuEPO (Example 7A) was mixed with the plasmid pMG2 containing a mouse dihydrofolate reductase gene cloned into the bacterial plasmid vector pBR322 (per Gasser, et al., *supra*.) The plasmid mixture and carrier DNA was transfected into CHO DHFR- cells. (Cells which acquire one plasmid will generally also acquire a second plasmid). After three days, the cells were dispersed by trypsinization into several 100 mm culture plates in media lacking hypoxanthine and thymidine. Only those cells which have been stably transformed with the DHFR gene, and thereby the EPO gene, survive in this media. After 7—21 days, colonies of surviving cells became apparent. These transformant colonies, after dispersion by trypsinization can be continuously propagated in media lacking hypoxanthine and thymidine, creating new cell strains (e.g., CHO pDSVL—MkEPO, CHO pSVgHuEPO, CHO—pDSVL—gHuEPO).

35 Culture fluids from the above cell strains were tested in the RIA for the presence of recombinant monkey or human EPO. Media for strain CHO pDSVL—MkEPO contained EPO with immunological properties like that obtained from COS—1 cells transfected with plasmid pDSVL—MkEPO. A representative 65 hour culture fluid contained monkey EPO at 0.60 U/ml.

40 Culture fluids from CHO pSVgHuEPO and CHO pDSVL-gHuEPO contained recombinant human EPO with immunological properties like that obtained with COS—1 cells transfected with plasmid pSVgHuEPO or pDSVL—gHuEPO. A representative 3 day culture fluid from CHO pSVgHuEPO contained 2.99 U/ml of human EPO and a 5.5 day sample from CHO pDSVL—gHuEPO had 18.2 U/ml of human EPO as measured by the RIA.

45 The quantity of EPO produced by the cell strains described above can be increased by gene amplification giving new cell strains of greater productivity. The enzyme dihydrofolate reductase (DHFR) which is the product coded for by the DHFR gene can be inhibited by the drug methotrexate (MTX). More specifically, cells propagated in media lacking hypoxanthine and thymidine are inhibited or killed by MTX. Under the appropriate conditions, (e.g., minimal concentrations of MTX) cells resistant to and able to grow in MTX can be obtained. These cells are found to be resistant to MTX due to an amplification of the number of their DHFR genes, resulting in increased production of DHFR enzyme. The surviving cells can, in turn, be treated with increasing concentrations of MTX, resulting in cell strains containing greater numbers of DHFR genes. "Passenger genes" (e.g., EPO) carried on the expression vector along with the DHFR gene or transformed with the DHFR gene are frequently found also to be increased in their gene copy number.

As examples of practice of this amplification system, cell strain CHO pDSVL—MkE was subjected to increasing MTX concentrations (0 nM, 30 nM and 100 nM). Representative 65-hour culture media samples from each amplification step were assayed by RIA and determined to contain 0.60, 2.45 and 6.10 U/ml, respectively. Cell strain CHO pDSVL—gHuEPO was subjected to a series of increasing MTX concentrations of 30 nM, 50 nM, 100 nM, 200 nM, 1  $\mu$ M, and 5  $\mu$ M MTX. A representative 3-day culture media sample from the 100 nM MTX step contained human EPO at  $3089 \pm 129$  U/ml as judged by RIA. Representative 48 hour cultural medium samples from the 100 nM and 1  $\mu$ M MTX steps contained, respectively, human EPO at 466 and 1352 U/ml as judged by RIA (average of triplicate assays). In these procedures,  $1 \times 10^6$  cells were plated in 5 ml of media in 60 mm culture dishes. Twenty-four hours later the media were removed and replaced with 5 ml of serum-free media (high glucose DMEM supplemented with 0.1 mM non-essential amino acids and L-glutamine). EPO was allowed to accumulate for 48 hours in the serum-free media. The media was collected for RIA assay and the cells were trypsinized and counted. The average RIA values of 467 U/ml and 1352 U/ml for cells grown at 100 nM and 1  $\mu$ M MTX, respectively, provided actual yields of 2335 U/plate and 6750 U/plate. The average cell numbers per plate were  $1.94 \times 10^6$  and  $3.12 \times 10^6$  cells, respectively. The effective production rates for these culture conditions were thus 1264 and 2167 U/10 $^6$  cells/48 hours.

The cells in the cultures described immediately above are a genetically heterogeneous population. Standard screening procedures are being employed in an attempt to isolate genetically homogeneous clones with the highest production capacity. See, Section A, Part 2, of "Points to Consider in the Characterization of Cell Lines Used to Produce Biologics", June 1, 1984, Office of Biologics Research Review, Center for Drugs and Biologics, U.S. Food and Drug Administration.

The productivity of the EPO producing CHO cell lines described above can be improved by appropriate cell culture techniques. The propagation of mammalian cells in culture generally requires the presence of serum in the growth media. A method for production of erythropoietin from CHO cells in media that does not contain serum greatly facilitates the purification of erythropoietin from the culture medium. The method described below is capable of economically producing erythropoietin in serum-free media in large quantities sufficient for production.

Strain CHO pDSVL—gHuEPO cells, grown in standard cell culture conditions, are used to seed spinner cell culture flasks. The cells are propagated as a suspension cell line in the spinner cell culture flask in media consisting of a 50—50 mixture of high glucose DMEM and Ham's F12 supplemented with 5% fetal calf serum, L-glutamine, Penicillin and Streptomycin, 0.05 nM non-essential amino acids and the appropriate concentration of methotrexate. Suspension cell culture allows the EPO-producing CHO cells to be expanded easily to large volumes. CHO cells, grown in suspension, are used to seed roller bottles at an initial seeding density of  $1.5 \times 10^7$  viable cells per 850 cm $^2$  roller bottle in 200 ml of media. The cells are allowed to grow to confluence as an adherent cell line over a three-day period. The media used for this phase of the growth is the same as used for growth in suspension. At the end of the three-day growth period, the serum containing media is removed and replaced with 100 ml of serum-free media; 50—50 mixture of high glucose DMEM and Ham's F12 supplemented with 0.05 mM non-essential amino acids and L-glutamine. The roller bottles are returned to the roller bottle incubator for a period of 1—3 hours and the media again is removed and replaced with 100 ml of fresh serum-free media. The 1—3 hour incubation of the serum-free media reduces the concentration of contaminating serum proteins. The roller bottles are returned to the incubator for seven days during which erythropoietin accumulates in the serum-free culture media. At the end of the seven-day production phase, the conditioned media is removed and replaced with fresh serum-free medium for a second production cycle. As an example of the practice of this production system, a representative seven-day, serum-free media sample contained human erythropoietin at  $3892 \pm 409$  U/ml as judged by the RIA. Based on an estimated cell density of 0.9 to  $1.8 \times 10^5$  cells/cm $^2$ , each 850 cm $^2$  roller bottle contained from 0.75 to  $1.5 \times 10^8$  cells and thus the rate of production of EPO in the 7-day, 100 ml culture was 750 to 1470 H/10 $^6$  cells/48 hours.

Culture fluids from cell strain CHO pDSVL—MkEPO carried in 10 nM MTX were subjected to RIA *in vitro* and *in vivo* EPO activity assays. The conditioned media sample contained  $41.2 \pm 1.4$  U/ml of MkEPO as measured by the RIA,  $41.2 \pm 0.064$  U/ml as measured by the *in vitro* biological activity assay and  $42.5 \pm 5$  U/ml as measured by the *in vivo* biological activity assay. Amino acid sequencing of polypeptide products revealed the presence of EPO products, a principle species having 3 residues of the "leader" sequence adjacent the putative amino terminal alanine. Whether this is the result of incorrect membrane processing of the polypeptide in CHO cells or reflects a difference in structure of the amino terminus of monkey EPO *vis-a-vis* human EPO, is presently unknown.

Culture fluids from cell strain CHO pDSVL—gHuEPO were subjected to the three assays. A 5.5 day sample contained recombinant human EPO in the media at a level of 18.2 U/ml by RIA assay,  $15.8 \pm 4.6$  U/ml by *in vitro* assay and  $16.8 \pm 3.0$  U/ml by *in vivo* assay.

Culture fluid from CHO pDSVL—gHuEPO cells prepared amplified by stepwise 100 nM MTX were subjected to the three assays. A 3.0 day sample contained recombinant human EPO at a level of  $3089 \pm 129$  U/ml by RIA,  $2589 \pm 71.5$  U/ml by *in vitro* assay, and  $2040 \pm 160$  U/ml by *in vivo* assay. Amino acid sequencing of this product reveals an amino terminal corresponding to that designated in Table VI.

Cell conditioned media from CHO cells transfected with plasmid pDSVL—MkE in 10 nM MTX were pooled, and

the MTX dialyzed out over several days, resulting in media with an EPO activity of  $221 \pm 5.1$  U/ml (EPO—CCM). To determine the *in vivo* effect of the EPO—CCM upon hematocrit levels in normal Balb/C mice, the following experiment was conducted. Cell conditioned media from untransfected CHO cells (CCM) and EPO—CCM were adjusted with PBS. CCM was used for the control group (3 mice) and two dose levels of EPO—CCM — 4 units per injection and 44 units per injection — were employed for the experimental groups (2 mice/group). Over the course of 5 weeks, the seven mice were injected intraperitoneally, 3 times per week. After the eighth injection, average hematocrit values for the control group were determined to be 50.4%; for the 4U group, 55.1%; and, for the 44U group, 67.9%.

Mammalian cell expression products may be readily recovered in substantially purified form from culture media using HPLC (C<sub>4</sub>) employing an ethanol gradient, preferably at pH7.

A preliminary attempt was made to characterize recombinant glycoprotein products from conditioned medium of COS—1 and CHO cell expression of the human EPO gene in comparison to human urinary EPO isolates using both Western blot analysis and SDS—PAGE. These studies indicated that the CHO-produced EPO material had a somewhat higher molecular weight than the COS—1 expression product which, in turn, was slightly larger than the cooled source human urinary extract. All products were somewhat heterogeneous. Neuraminidase enzyme treatment to remove sialic acid resulted in COS—1 and CHO recombinant products of approximately equal molecular weight which were both nonetheless larger than the resulting asialo human urinary extract. Endoglycosidase F enzyme (EC 3.2.1) treatment of the recombinant CHO product and the urinary extract product to totally remove carbohydrate from both resulted in substantially homogeneous products having essentially identical molecular weight characteristics.

Glycoprotein products provided by the present invention are thus comprehensive of products having a primary structural conformation sufficiently duplicative of that of a naturally-occurring erythropoietin to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring erythropoietin.

#### Example 11

The present example relates to the total manufacture by assembly of nucleotide bases of two structural genes encoding the human species EPO sequence of Table VI and incorporating, respectively "preferred" codons for expression in *E.coli* and yeast (*S.cerevisiae*) cells. Also described is the construction of genes encoding analogs of human EPO. Briefly stated, the protocol employed was generally as set out in the previously noted disclosure of Alton, et al. (WO 83/04053). The genes were designed for initial assembly of component oligonucleotides into multiple duplexes which, in turn, were assembled into three discrete sections. These sections were designed for ready amplification and, upon removal from the amplification system, could be assembled sequentially or through a multiple fragment ligation in a suitable expression vector.

Tables VIII through XIV below illustrate the design and assembly of a manufactured gene encoding a human EPO translation product lacking any leader or presequence but including an initial methionine residue at position -1. Moreover, the gene incorporated in substantial part *E.coli* preference codons and the construction was therefore referred to as the "ECEPO" gene.

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TABLE VIII

ECEPO SECTION 1 OLIGONUCLEOTIDES

5 1. AATTCTAGAAACCATGAGGGTAATAAAATA  
 2. CCATTATTTATTACCCATGGTTCTAG  
 10 3. ATGGCTCCGCCGCGTCTGATCTGCGAC  
 4. CTCGAGTCGAGATCAGACGGCGGAG  
 15 5. TCGAGAGTTCTGGAACGTTACCTGCTG  
 6. CTTCCAGCAGGTAAACGTTCCAGAACT  
 7. GAAGCTAAAGAAGCTGAAACATC  
 20 8. GTGGTGATGTTTCAGCTTCTTTAG  
 9. ACCACTGGTTGTGCTGAAACACTGTT  
 10. CAAAGAACAGTGTTCAGCACAAACCA  
 25 11. TTTAACGAAAACATTACGGTACCG  
 12. GATCCGGTACCGTAATGTTTCGTT

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TABLE IX

ECEPO SECTION 1

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XbaI

EcoRI 1 AATTCTAG AAACCATGAG 1 GGTAATAAAA 1 TA1 ATGGCTCC 3 GCGCGTCTG  
 GATC TTTGGTACTC CCATTATTT ATTAC1 GAGG CGGCGCAGAC  
 40 2 4

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ATCTGGACT 5 CGAGAGTCT 5 GGAACGTTAC CTGCTG6 AAG CTAAGAAC  
 TAGACGCTGA GCTCTCAAGA CCTTGCAATG GACGACCT6 GATTCTT6 CG  
 45 6

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TGAAAACATC 7 ACCACTGGTT 9 GTGCTGAACA CTGTT10 TTG AACGAAAC  
 ACTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAC 11 TTGCTTTGT  
 50 8 10 11

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KpnI 8 BamHI  
 TTACGGTACC G  
 AATGCCATGG CCTAG  
 55 12

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TABLE X  
ECEPO SECTION 2 OLIGONUCLEOTIDES

5. 1. AATTGGTACCAAGACACCAAGGT

10. 2. GTTAACCTTGGTGTCTGGTACCG

3. TAACTTCTACCGCTTGGAAACGTAT

15. 4. TTCCATACGTTCCAAGCGTAGAA

5. GGAAGTTGGTCAACAAGCAGTTGAAGT

16. 6. CCAAACCTCAACTGCTTGTGACCAAC

7. TTGGCAGGGTCTGGCACTGCTGAGCG

20. 8. GCCTCGCTCAGCAGTGCCAGACCCCTG

9. AGGCTGTACTGCCTGGCCAGTACA

25. 10. GCAGTGCCTGGCCACGCAGTACA

11. CTGCTGGTAAACTCCTCTCAGCCGT

12. TTCCCACGGCTGAGAGGGAGTTACCA

13. GGGAAACCGCTGCAGCTGCATGTTGAC

30. 14. GCTTTGTCAACATGCAGCTGCAGCGG

15. AAAGCAGTATCTGGCCTGAGATCTG

16. GATCCAGATCTCAGGCCAGATACT

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TABLE XI  
ECEPO SECTION 2

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TABLE XII  
ECEPO SECTION 3

5	1. GATCCAGATCTCTGACTACTCTGC
	2. ACGCAGCAGAGTAGTCAGAGATCTG
10	3. TGCCTGCTCTGGGTGCACAGAAAGAGG
	4. GATAGCCTCTTCTGTGCACCCAGAGC
	5. CTATCTCTCCGCCGGATGCTGCATCT
15	6. CAGCAGATGCAGCATCCGGCGGAGA
	7. GCTGCACCGCTGGTACCATCACTG
20	8. ATCAGCAGTGATGGTACGCAGCGGTG
	9. CTGATACTTCCGCAAACGTGTTCG
	10. ATACACGAAACAGTTGCCGAAGGT
25	11. TGTATACTCTAACTTCCCTGGTGGTA
	12. CAGTTTACCAACGCAGGAAGTTAGAGT
	13. AACTGAAACTGTATACTGGCGAAGC
30	14. GGCATGCTTCGCCAGTATAACAGTTT
	15. ATGCCGTACTGGTGACCGCTAATAG
35	16. TCGACTATTAGCGGTACCCAGTAC

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TABLE XIII  
ECEPO SECTION 3

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BamHI SaI  
GA TCCAGATCTCTG  
GTCTAGAGAC

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ACTACTCTGC 1 TGCGTGCTCT 3 GGGTGCACAG AAAGAGGCTA TCTCTCCGCC  
TGATGAGAGCG ACCGACGGAGA CCCACGTGTC TTTCTCCGAT AGAGAGGGCGG  
2 4

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GGATGCTGCA TCT7 GCTGCAC CGCTGCGTAC CATCACTGT GATAACCT9  
CCTACGACGT AGACGACGT GCGACGCGATG GTAGTGACGA CTATGGAAAGG  
6 8

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GCAAAC11 TCGGTTATAC TCT10ACTTCC TGCGTGGTA 13 ACTGAAACTG  
CGTTGACAA AGCACATA12 AGATTGAAGG AGCCACCA14 TTGACTTGAC

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TABLE XIV  
ECEPO GENE

5 XbaI -1 1  
 CTAG AAACCATGAG GGTAAATAAAA TAATGGCTCC GCCCGCTCTG  
 TTTGGTACTC CCATTATTT ATTACCGAGG CGGGCGCAGAC  
 10 ATCTGCGACT CGAGAGTTCT GGAACGTTAC CTGCTGGAAG CAAAGAAC  
 TAGACGCTGA GCTCTCAAGA CCTTGCATG GACGACCTTC GATTCTTCC  
 15 TGAAAACATC ACCACTGGTT GTGCTGAACA CTGTTCTTG AACGAAAACA  
 ACTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAC TTGCTTTGT  
 20 TTACGGTACC AGACACCAAG GTTAACTTCT ACGCTTGGAA ACGTATGGAA  
 AATGCCATGG TCTGTGGTTC CAATTGAAGA TGCACACCTT TGCATACCTT  
 25 GTTGGTCAAC AAGCAGTTGA AGTTTGGCAG GGTCTGGCAC TGCTGAGCGA  
 CAACCAGTTG TTCGTCAACT TCAAACCGTC CCAGACCGTG ACGACTCGCT  
 30 GGCTGTACTG CGTGGCCAGG CACTGCTGGT AAACCTCTCT CAGCCGTGGG  
 CCGACATGAC GCACCCGTCC GTGACGACCA TTGAGGGAGA GTCGGCACCC  
 35 AACCGCTGCA GCTGCATGTT GACAAAGCAG TATCTGGCCT GAGATCTCTG  
 TTGGCGACGT CGACGTACAA CTGTTCGTC ATAGACCGGA CTCTAGAGAC  
 40 ACTACTCTGC TGCCTGCTCT GGGTGCACAG AAAGAGGCTA TCTCTCCGCC  
 TGATGAGACG ACGCACGAGA CCCACGTGTC TTTCTCCGAT AGAGAGGCGG  
 45 GGATGCTGCA TCTGCTGCAC CGCTGCGTAC CATCACTGCT GATACTTCC  
 CCTACGACGT AGACGACGTG GCGACGATG GTAGTACGA CTATGGAAGG  
 50 GCAAACGTGTT TCGTGTATAC TCTAACTTCC TGGCTGGTAA ACTGAAACTG  
 CGTTTGACAA AGCACATATG AGATTGAAGG ACGCACCAATT TGACTTTGAC  
 55 TATACTGGCG AAGCATGCCG TACTGGTGAC CGCTAAATAG SalI  
 ATATGACCGC TTGCTACGGC ATGACCACTG GCGATTATCA GCT

50 More particularly, Table VIII illustrates oligonucleotides employed to generate the Section 1 of the ECEPO gene  
 encoding amino terminal residues of the human species polypeptide. Oligonucleotides were assembled into duplexes  
 (1 and 2, 3 and 4, etc.) and the duplexes were then ligated to provide ECEPO Section 1 as in Table IX. Note that the  
 assembled section includes respective terminal *Eco*RI and *Bam*HI sticky ends, that "downstream" of the *Eco*RI sticky  
 end is a *Xba*I restriction enzyme recognition site; and that "upstream" of the *Bam*HI sticky end is a *Kpn*I recognition  
 site. Section 1 could readily be amplified using the M13 phage vector employed for verification of sequence of the  
 section. Some difficulties were encountered in isolating the section as an *Xba*I/*Kpn*I fragment from RF DNA generated  
 in *E. coli*, likely due to methylation of the *Kpn*I recognition site bases within the host. Single-stranded phage DNA was  
 therefore isolated and rendered into double-stranded form *in vitro* by primer extension and the desired double-stranded

fragment was thereafter readily isolated.

ECEPO gene Sections 2 and 3 (Tables XI and XIII) were constructed in a similar manner from the oligonucleotides of Tables X and XII, respectively. Each section was amplified in the M13 vector employed for sequence verification and was isolated from phage DNA. As is apparent from Table XI, ECEPO Section 2 was constructed with *Eco*RI and *Bam*HI sticky ends and could be isolated as a *Kpn*I/*Bgl*II fragment. Similarly, ECEPO Section 3 was prepared with *Bam*HI and *Sai*I sticky ends and could be isolated from phage RF DNA as a *Bgl*II/*Sai*I fragment. The three sections thus prepared can readily be assembled into a continuous DNA sequence (Table XIV) encoding the entire human species EPO polypeptide with an amino terminal methionine codon (ATG) for *E. coli* translation initiation. Note also that "upstream" of the initial ATG is a series of base pairs substantially duplicating the ribosome binding site sequence of the highly expressed OMP—f gene of *E. coli*.

Any suitable expression vector may be employed to carry the ECEPO. The particular vector chosen for expression of the ECEPO gene as the "temperature sensitive" plasmid pCFM536 — a derivative of plasmid pCFM414 (A.T.C.C. 40076) — as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, by Charles F. Morris. More specifically, pCFM536 was digested with *Xba*I and *Hind*III; the large fragment was isolated and employed in a two-part ligation with the ECEPO gene. Sections 1 (*Xba*I/*Kpn*I), 2 (*Kpn*I/*Bgl*II) and 3 (*Bgl*II/*Sai*I) had previously been assembled in the correct order in M13 and the EPO gene was isolated therefrom as a single *Xba*I/*Hind*III fragment. This fragment included a portion of the polylinker from M13 mp9 phage spanning the *Sai*I to *Hind*III sites therein. Control of expression in the resulting expression plasmid, p536, was by means of a lambda *P<sub>L</sub>* promoter, which itself may be under control of the *C<sub>1857</sub>* repressor gene (such as provided in *E. coli* strain K12ΔHtrp).

The manufactured ECEPO gene above may be variously modified to encode erythropoietin analogs such as [Asn<sup>2</sup>, des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO and [His<sup>7</sup>]hEPO, as described below.

A. [Asn<sup>2</sup>, des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO

Plasmid 536 carrying the ECEPO manufactured gene of Table XIV as a *Xba*I to *Hind*III insert was digested with *Hind*III and *Xba*I. The latter endonuclease cuts the ECEPO gene at a unique, 6 base pair recognition site spanning the last base of the codon encoding Asp<sup>8</sup> through the second base of the Arg<sup>10</sup> codon. A *Xba*I/*Xba*I "linker" sequence was manufactured having the following sequence:

*Xba*I      +1    2    7    8    9  
 Met Ala Asn Cys Asp      *Xba*I  
 5' -CTAG ATG GCT AAT TGC GAC-3',  
 3' -TAC CGA TTA ACG CTG AGCT-5'

The *Xba*I/*Xba*I linker and the *Xba*I/*Hind*III ECEPO gene sequence fragment were inserted into the large fragment resulting from *Xba*I and *Hind*III digestion of plasmid pCFM526 — a derivative of plasmid pCFM414 (A.T.C.C. 40076) — as described in co-pending U.S. Patent Application Serial No. 636,727, filed August 6, 1984, by Charles F. Morris, to generate a plasmid-borne DNA sequence encoding *E. coli* expression of the Met<sup>1</sup> form of the desired analog.

B. [His<sup>7</sup>]hEPO

Plasmid 536 was digested with *Hind*III and *Xba*I as in part A above. A *Xba*I/*Xba*I linker was manufactured having the following sequence:

*Xba*I      +1    2    3    4    5    6    7    8    9      *Xba*I  
 Met Ala Pro Pro Arg Leu Ile His Asp  
 5' -CTAG ATG GCT CCG CCA CGT CTG ATC CAT GAC-3',  
 3' -TAC CGA GGC GGT GCA GAC TAG GTA CTG AGCT-5'

The linker and the *Xba*I/*Hind*III ECEPO sequence fragment were then inserted into pCFM526 to generate a plasmid-borne DNA sequence encoding *E. coli* expression of the Met<sup>1</sup> form of the desired analog.

Construction of a manufactured gene ("SCEPO") incorporating yeast preference codons is as described in the following Tables XV through XXI. As was the case with the ECEPO gene, the entire construction involved formation of three sets of oligonucleotides (Tables XV, XVII and XIX) which were formed into duplexes and assembled into sections (Tables XVI, XVIII and XX). Note that synthesis was facilitated in part by use of some sub-optimal codons in both the SCEPO and ECEPO constructions, i.e., oligonucleotides 7—12 of Section 1 of both genes were identical, as were

oligonucleotides 1—6 of Section 2 in each gene.

TABLE XV

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SCEPO SECTION 1 OLIGONUCLEOTIDES

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1. AATTCAAGCTTGGATAAAAAGAGCT
2. GTGGAGCTCTTTATCCAAGCTTG
3. CCACCAAGATTGATCTGTGACTC
4. TCTCGAGTCACAGATCAATCTTG
5. GAGAGTTTGGAAAGATACTTGTG
6. CTTCCAACAAGTATCTTCCAAAC
7. GAAGCTAAGAAGCTGAAAACATC
8. GTGGTGTGTTCAAGCTTCTTAG
9. ACCACTGGTTGTGCTGAACACTGTT
10. CAAAGAACAGTGTTCAGCACAAACCA
11. TTTGAACGAAAACATTACGGTACCG
12. GATCCGGTACCGTAATGTTTCGTT

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TABLE XVI

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SCEPO SECTION 1

EcoRI HindIII 1  
AATTCA AGCTTGGATA  
GT TCGAACCTAT

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AAAGAGCTCC ACCAACGATTG ATCTGTGACT CGAGAGTTT  
TTTCTCGAGG TGGTTCTAAC TAGACACTGA GCTCTCAAAA

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5 GGAAAGATAC TTGTTGAAAG CTAAGAACG TGAAAACATC ACCACTGGTT  
CCTTTCTATG AACAAACCTTC GATTTCTCG ACTTTGTAG TGGTACCAA

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9 GTGCTGAACA CTGTTCTTG AACGAAACAA TTACGGTACC G KpnI 8amHI  
CACGACTTGT GACAAGAAAC TTGCTTTGT AATGCCATGG CCTAG

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TABLE XVIISCEPO SECTION 2 OLIGONUCLEOTIDES

5

1. AATTCCGGTACCAAGACACCAAGGT
10. GTTAACCTTGGTGTCTGGTACCG
3. TAACTTCTACGCTTGGAAACGTAT
4. TTCCATACGTTCCAAGCGTAGAA
15. GGAAGTTGGTCAACAAGCAGTTGAAGT
6. CCAAACTTCAACTGCTTGTGACCAAC
20. TTGGCAAGGTTGGCCTTGTATCTG
8. GCTTCAGATAACAAGGCCAAACCTG
9. AAGCTGTTTGAGAGGTCAAGCCT
25. AACAAAGGCTTGACCTCTCAAAACA
11. TGTGGTTAACTCTTCTCAACCATGGG
12. TGGTCCCAGGTTGAGAAGAGTTAAC
30. 13. AACCATTGCAATTGCACGTCGAT
14. CTTTATCGACGTGCAATTGCAA
35. 15. AAAGCCGTCTGGTTGAGATCTG
16. GATCCAGATCTCAAACCAAGAGACGG

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TABLE XVIII  
SCEPO SECTION 2

5 KpnI  
EcoRI 1  
A ATTCGGTACCC AGACACCAAG  
GCCATGG TCTGTGGTTC  
10 2  
  
3 15 GTTAACTTCT ACGCTTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTGAA  
CAATTGAGA TGCGAACCTT TGCATACCTT CAACCAGTTG TTCGACAACT  
16 4 6  
  
7 20 AGTTTGGCAA GGTTTGGCCT TGTATCTG AGCTGTTTTG AGAGGTCAAG  
TCAAACCCGTT CCAAACCCGGA ACAATAGACT TCGACAAAC TCTCCAGTTC  
8 10  
  
11 25 CCTTTGGT TAACTCTCT CAACCATGGG ACCATGCA ATTGCACGTC  
GGAACAACCA ATTGAGAAGA GTTGGTACCC TGGTACGT TAACGTGCAG  
12 13 14  
  
15 30 GATAAAGCCG TCTCTGGTT GAGATCTG BalII BamHI  
CTATTTCGGC AGAGACCAAA CTCTAGACCTA G  
16

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TABLE XIX

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SCEPO SECTION 3 OLIGONUCLEOTIDES

10

1. GATCCAGATCTTGACTACTTGTT
2. TCTCAACAAAGTAGTCAAAGATCTG
3. GAGAGCTTGGGTGCTCAAAAGGAAG
4. ATGGCTTCCTTGTGAGCACCCAAAGC
5. CCATTTCCCCACCAGACGGCTGCTT
6. GCAGAAGCAGCGTCTGGTGGGGAA
7. CTGCCGCTCCATTGAGAACCATC
8. CAGTGATGGTTCTCAATGGAGCG
9. ACTGCTGATACTTCAGAAAGTT
10. GAATAACTTCTGAAGGTATCAG
11. ATTCAAGAGTTACTCCAACTTCT
12. CTCAAGAAGTTGGAGTAAACTCT
13. TGAGAGGTAAATTGAAGTTGTACAC
14. ACCGGTGTACAACCTCAATTACCT
15. CGGTCAAGCCTGTAGAACTGGT
16. CTGTCACCAGTTCTACAGGGCTTC
17. GACAGATAAGCCCCGACTGATAA
18. GTTGTATCAGTCGGGCTTAT
19. CAACAGTGTAGATGTAACAAAG
20. TCGACTTGTACATCTACACT

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TABLE XX  
SCEPO SECTION 3

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BamHI BalII 1  
 GATC CAGATCTTGT ACTACTTTGT TAGAGCTTT  
 GTCTAGAAAC TGATGAAACA ACTCTGAAA  
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3 GGGTGTCAA AAGGAAGCCA TTTCCCACC AGACGCTGCT TCTGCCGCTC  
 CCCACGAGTT TTCCTTCGGT AAGGGGTGG TCTGCGACGA AGACGGCCAG  
 4 5 6

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7 CATTGAGAAC CATACTGCT GATACTTCA GAAAGTTTT CAGAGTTAC  
 GTAACTCTTG GTAGTGACGA CTATGAAGT CTTCAATAA GTCTCAAATG  
 8 9 10 11

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TCCAACTTCT TGAGAGGTAA ATTGAAGTTG TACACGGTG AAGCCCTGTAG  
 AGGTTGAAGA ACTCTCCATT TAACTCAAC ATGTGGCCAC TTCGGACATC  
 13 14 15 16

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AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG  
 TTGACCACTG TCTATTCGGG CTGACTATTG TTGCACATC  
 17 18 19

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SalI  
 ATGTAAAAA G  
 TACATTGTTT CAGCT  
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TABLE XXI  
SCEPO GENE

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-1 +1

HindIII ArgAla

AGCTTGGATA AAAGAGCTCC ACCAAGATTG ATCTGTGACT CGAGAGTTT  
ACCTAT TTTCTCGAGG TGTTTCTAAC TAGACACTGA GCTCTCAAAA

10

GGAAAGATAAC TTGTTGGAAG CTAAAGAACG TGAAAACATC ACCACTGGTT  
CCTTTCTATG AACAAACCTTC GATTTCTCG ACTTTTGTAG TGGTGACCAA

15

GTGCTGAACA CTGTTCTTTG AACGAAAACA TTACGGTACC AGACACCAAG  
CACGACTTGT GACAAGAAAC TTGCTTTGT AATGCCATGG TCTGTGGTTC

20

GTAACTTCT ACACCTGGAA ACGTATGGAA GTTGGTCAAC AAGCTGTTGA  
CAATTGAAGA TGCGAACCTT TGCAACCTT CAACCAAGTTG TTCGACAACT

AGTTTGGCAA GGTTTGGCCT TGTTATCTGA AGCTGTTTG AGAGGTCAAG  
TCAAAACCGTT CCAAACCGGA ACAATAGACT TCGACAAAAC TCTCCAGTTC

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CCTTGTGGT TAACTCTTCT CAACCATGGG AACCAATTGCA ATTGCACGTC  
GGAACAAACCA ATTGAGAAGA GTTGGTACCC TTGGTAACGT TAACGTGCAG

30

GATAAAGCCG TCTCTGGTTT GAGATCTTG ACTACTTTGT TGAGAGCTT  
CTATTCGGC AGAGACCAAA CTCTAGAAAC TGATGAAACA ACTCTCGAAA

35

GGGTGCTCAA AAGGAAGCCA TTTCCCCACC AGACGCTGCT TCTGCCGCTC  
CCCACGAGTT TTCCCTCGGT AAAGGGGTGG TCTGCGACGA AGACGGCGAG

CATTGAGAAC CATCACTGCT GATAACCTCA GAAAGTTATT CAGAGTTAC  
GTAACCTTG GTAGTGACGA CTATGAAAGT CTTCAATAA GTCTCAAATG

40

TCCAACCTCT TGAGAGGTAA ATTGAAGTTG TACACCGGTG AAGCCTGTAG  
AGGTTGAAGA ACTCTCCATT TAACTTCAC ACTGTGGCCAC TTCGGACATC

45

AACTGGTGAC AGATAAGCCC GACTGATAAC AACAGTGTAG  
TTGACCACTG TCTATTGGGG CTGACTATTG TTGTCACATC

50

SalI  
ATGTAACAAA G  
TACATTGTTT CAGCT

55

The assembled SCEPO sections were sequenced in M13 and Sections 1, 2 and 3 were isolatable from the phage as *HindIII/KpnI/BglI*, and *BglII/SalI* fragments.

The presently preferred expression system for SCEPO gene products is a secretion system based on *S.cerevisiae*  $\alpha$ -factor secretion, as described in co-pending U.S. Patent Application Serial No. 487,753, filed April 22, 1983, by Grant A. Bitter, published October 31, 1984 as European Patent Application 0 123 294. Briefly put, the system involves

constructions wherein DNA encoding the leader sequence of the yeast  $\alpha$ -factor gene product is positioned immediately 5' to the coding region of the exogenous gene to be expressed. As a result, the gene product translated includes a leader or signal sequence which is "processed off" by an endogenous yeast enzyme in the course of secretion of the remainder of the product. Because the construction makes use of the  $\alpha$ -factor translation initiation (ATG) codon, there was no need to provide such a codon at the -1 position of the SCEPO gene. As may be noted from Table XXI, the alanine (+1) encoding sequence is preceded by a linker sequence allowing for direct insertion into a plasmid including the DNA for the first 80 residues of the  $\alpha$ -factor leader following the  $\alpha$ -factor promoter. The specific preferred construction for SCEPO gene expression involved a four-part ligation including the above-noted SCEPO section fragments and the large fragment of *Hind*III/ *Sal*I digestion of plasmid pAC3. From the resulting plasmid pAC3/SCEPO, the  $\alpha$ -factor promoter and leader sequence and SCEPO gene were isolated by digestion with *Bam*HI and ligated into *Bam*HI digested plasmid pYE to form expression plasmid pYE/SCEPO.

## Example 12

15 The present example relates to expression of recombinant products of the manufactured ECEPO and SCEPO genes within the expression systems of Example 11.

In use of the expression system designed for use of *E.coli* host cells, plasmid p536 of Example 11 was transformed into AM7 *E.coli* cells previously transformed with a suitable plasmid, pMW1, harboring a  $C_{1857}$  gene. Cultures of cells in LB broth (Ampicillin 50  $\mu$ g/ml and kanamycin 5  $\mu$ g/ml, preferably with 10 mM  $MgSO_4$ ) were maintained at 28°C and upon growth of cells in culture to  $O.D._{600} = 0.1$ , EPO expression was induced by raising the culture temperature to 42°C. Cells grown to about 40 O.D. provided EPO production (as estimated by gel) of about 5 mg/OD liter.

Cells were harvested, lysed, broken with French Press (10,000 psi) and treated with lysozyme and NP—40 detergent. The pellet resulting from 24,000 xg centrifugation was solubilized with guanidine HCl and subjected to further purification in a single step by means of  $C_4$  (Vydac) Reverse Phase HPLC (EtOH, 0—80%, 50 mM  $NH_4Ac$ , pH 4.5). Protein sequencing revealed the product to be greater than 95% pure and the products obtained revealed two different amino terminals A—P—P—R... and P—P—R... in a relative quantitative ratio of about 3 to 1. This latter observation of hEPO and [des Ala<sup>1</sup>]hEPO products indicates that amino terminal "processing" within the host cells serves to remove the terminal methionine and in some instances the initial alanine. Radioimmunoassay activity for the isolates was at a level of 150,000 to 160,000 U/mg; *in vitro* assay activity was at a level of 30,000 to 62,000 U/mg; and *in vivo* assay activity ranged from about 120 to 720 U/mg. (Cf., human urinary isolate standard of 70,000 U/mg in each assay.) The dose response curve for the recombinant product in the *in vivo* assay differed markedly from that of the human urinary EPO standard.

The EPO analog plasmids formed in parts A and B of Example 11 were each transformed into pMW1-transformed AM7 *E.coli* cells and the cells were cultured as above. Purified isolates were tested in both RIA and *in vitro* assays. RIA and *in vitro* assay values for [Asn<sup>2</sup>, des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO expression products were approximately 11,000 U/mg and 6,000 U/mg protein, respectively, while the assay values for [His<sup>7</sup>]hEPO were about 41,000 U/mg and 14,000 U/mg protein, respectively, indicating that the analog products were from one-fourth to one-tenth as "active" as the "parent" expression product in the assays.

In the expression system designed for use of *S.cerevisiae* host cells, plasmid pYE/SCEPO was transformed into two different strains, YSDP4 (genotype *a pep4—3 trp1*) and RK81 (genotype *aa pep4—3 trp1*). Transformed YSDP4 hosts were grown in SD medium (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 62 (1983) supplemented with casamino acids at 0.5%, pH 6.5 at 30°C. Media harvested when the cells had been grown to 36 O.D. contained EPO products at levels of about 244 Ug/ml (97  $\mu$ g/OD liter by RIA). Transformed RK81 cells grown to either 6.5 O.D. or 60 O.D. provided media with EPO concentrations of about 80—90 U/ml (34  $\mu$ g/OD liter by RIA). Preliminary analyses reveal significant heterogeneity in products produced by the expression system, likely to be due to variations in glycosylation of proteins expressed, and relatively high mannose content of the associated carbohydrate.

50 Plasmids pAC3 and pYE in HB101 *E.coli* cells were deposited in accordance with the Rules of Practice of the U.S. Patent Office on September 27, 1984, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under deposit numbers AT.C.C. 39881 and AT.C.C. 39882, respectively. Plasmids pCFM526 in AM7 cells, pCFM536 in JM103 cells, and pMW1 in JM103 cells were likewise deposited on November 21, 1984 as AT.C.C. 39932, 39934, and 39933, respectively. *Saccharomyces cerevisiae* strains YSDP4 and RK81 were deposited on November 21, 1984 as AT.C.C. 20734 and 20733, respectively.

55 It should be readily apparent from consideration of the above illustrative examples that numerous exceptionally valuable products and processes are provided by the present invention in its many aspects.

Polypeptides provided by the invention are conspicuously useful materials, whether they are microbially expressed products or synthetic products, the primary, secondary or tertiary structural conformation of which was first made known by the present invention.

As previously indicated, recombinant-produced and synthetic products of the invention share, to varying degrees, the *in vitro* biological activity of EPO isolates from natural sources and consequently are projected to have utility as substitutes for EPO isolates in culture media employed for growth of erythropoietic cells in culture. Similarly, to the extent that polypeptide products of the invention share the *in vivo* activity of natural EPO isolates they are conspicuously suitable for use in erythropoietin therapy procedures practiced on mammals, including humans, to develop any or all of the effects heretofore attributed *in vivo* to EPO, e.g., stimulation of reticulocyte response, development of ferrokinetic effects (such as plasma from turnover effects and marrow transit time effects), erythrocyte mass changes, stimulation of hemoglobin C synthesis (see, Eschbach, et al., *supra*) and, as indicated in Example 10, increasing hematocrit levels in mammals. Included within the class of humans treatable with products of the invention are patients generally requiring blood transfusions and including trauma victims, surgical patients, renal disease patients including dialysis patients, and patients with a variety of blood composition affecting disorders, such as hemophilia, sickle cell disease, physiologic anemias, and the like. The minimization of the need for transfusion therapy through use of EPO therapy can be expected to result in reduced transmission of infectious agents. Products of the invention, by virtue of their production by recombinant methods, are expected to be free of pyrogens, natural inhibitory substances, and the like, and are thus likely to provide enhanced overall effectiveness in therapeutic processes *vis-a-vis* naturally derived products. Erythropoietin therapy with products of the present invention is also expected to be useful in the enhancement of oxygen carrying capacity of individuals encountering hypoxic environmental conditions and possibly in providing beneficial cardiovascular effects.

A preferred method for administration of polypeptide products of the invention is by parenteral (e.g., IV, IM, SC, or IP) routes and the compositions administered would ordinarily include therapeutically effective amounts of product in combination with acceptable diluents, carriers and/or adjuvants. Preliminary pharmacokinetic studies indicate a longer half-life *in vivo* for monkey EPO products when administered IM rather than IV. Effective dosages are expected to vary substantially depending upon the condition treated but therapeutic doses are presently expected to be in the range of 0.1 (~7U) to 100 (~7000U)  $\mu$ g/kg body weight of the active material. Standard diluents such as human serum albumin are contemplated for pharmaceutical compositions of the invention, as are standard carriers such as saline.

Adjuvant materials suitable for use in compositions of the invention include compounds independently noted for erythropoietic stimulatory effects, such as testosterone, progenitor cell stimulators, insulin-like growth factor, prostaglandins, serotonin, cyclic AMP, prolactin and triiodothyronine, as well as agents generally employed in treatment of aplastic anemia, such as methenolene, stanozolol and nandrolone [see, e.g., Resegotti, et al., *Panminerva Medica*, 23, 243—248 (1981); McGonigle, et al., *Kidney Int.*, 25(2), 437—444 (1984); Pavlovic-Kantera, et al., *Expt. Hematol.*, 8(Supp. 8), 283—291 (1980); and Kurtz, *FEBS Letters*, 14a(1), 105—108 (1982)]. Also contemplated as adjuvants are substances reported to enhance the effects of, or synergize, erythropoietin or asialo-EPO, such as the adrenergic agonists, thyroid hormones, androgens and BPA [see, Dunn, "Current Concepts in Erythropoiesis", John Wiley and Sons (Chichester, England, 1983); Weiland, et al., *Blut*, 44(3), 173—175 (1982); Kalmanti, *Kidney Int.*, 22, 383—391 (1982); Shahidi, *New Eng. J. Med.*, 289, 72—80 (1973); Fisher, et al., *Steroids*, 30(6), 833—845 (1977); Urabe, et al., *J. Exp. Med.*, 149, 1314—1325 (1979); and Billat, et al., *Expt. Hematol.*, 10(1), 133—140 (1982)] as well as the classes of compounds designated "hepatic erythropoietic factors" [see, Naughton, et al., *Acta Haemat.*, 69, 171—179 (1983)] and "erythrotropins" [as described by Congote, et al. in Abstract 364, Proceedings 7th International Congress of Endocrinology (Quebec City, Quebec, July 1-7, 1984); Congote, *Biochem. Biophys. Res. Comm.*, 115(2), 447—483 (1983) and Congote, *Anal. Biochem.*, 140, 428—433 (1984)] and "erythrogenins" [as described in Rothman, et al., *J. Surg. Oncol.*, 20, 105—108 (1982)]. Preliminary screenings designed to measure erythropoietic responses of ex-hypoxic polycythemic mice pre-treated with either 5- $\alpha$ -dihydrotestosterone or nandrolone and then given erythropoietin of the present invention have generated equivocal results.

Diagnostic uses of polypeptides of the invention are similarly extensive and include use in labelled and unlabelled forms in a variety of immunoassay techniques including RIA's, ELISA's and the like, as well as a variety of *vitro* and *in vivo* activity assays. See, e.g., Dunn, et al., *Expt. Hematol.*, 11(7), 590—600 (1983); Gibson, et al., *Pathology*, 16, 155—156 (1984); Krystal, *Expt Hematol.*, 11(7), 649—660 (1983); Saito, et al., *Jap. J. Med.*, 23(1), 15—21 (1984); Nathan, et al., *New Eng. J. Med.*, 308(9), 520—522 (1983); and various references pertaining to assays referred to therein. Polypeptides of the invention, including synthetic peptides comprising sequences of residues of EPO first revealed herein, also provide highly useful pure materials for generating polyclonal antibodies and "banks" of monoclonal antibodies specific for differing continuous and discontinuous epitopes of EPO. As one example, preliminary analysis of the amino acid sequences of Table VI in the context of hydrophobicity according to Hopp, et al., *P.N.A.S. (U.S.A.)*, 78, pp. 3824-3828 (1981) and of secondary structures according to Chou, et al., *Ann. Rev. Biochem.*, 47, p. 251 (1978) revealed that synthetic peptides duplicative of continuous sequences of residues spanning positions 41-57 inclusive, 116-118 inclusive and 144-166 inclusive are likely to produce a highly antigenic response and generate useful monoclonal and polyclonal antibodies immunoreactive with both the synthetic peptide and the entire protein. Such antibodies are expected to be useful in the detection and affinity purification of EPO and EPO-related products but are not claimed herein.

Illustratively, the following three synthetic peptides were prepared:

(1) hEPO 41-57, V-P-D-T-K-V-N-F-Y-A-W-K-R-M-E-V-G;  
 (2) hEPO 116-128, K-E-A-I-S-P-P-D-A-A-S-A-A;  
 5 (3) hEPO 144-166, V-Y-S-N-F-L-R-G-K-L-K-L-Y-T-G-E-A-C-R-T-G-O-R,

Preliminary immunization studies employing the above-noted polypeptides have revealed a relatively weak positive response to hEPO 41—57, no appreciable response to hEPO 116—128, and a strong positive response to hEPO 144—166, as measured by capacity of rabbit serum antibodies to immunoprecipitate  $^{125}\text{I}$ -labelled human urinary EPO isolates. Preliminary *in vivo* activity studies on the three peptides revealed no significant activity either alone or in combination.

While the reduced sequences of amino acid residues of mammalian EPO provided by the illustrative examples essentially define the primary structural conformation of mature EPO, it will be understood that the specific sequence of 165 amino acid residues of monkey species EPO in Table V and the 166 residues of human species EPO in Table VI do not limit the scope of useful polypeptides provided by the invention. Comprehended by the present invention are those various naturally-occurring allelic forms of EPO which past research into biologically active mammalian polypeptides such as human  $\gamma$  interferon indicates are likely to exist. Compare, e.g., the human immune interferon species reported to have an arginine residue at position No. 140 in EPO published application 0 077 670 and the species reported to have glutamine at position No. 140 in Gray, et al., *Nature*, 295, pp. 503—508 (1982). Both species are characterized as constituting "mature" human  $\gamma$  interferon sequences.) Allelic forms of mature EPO polypeptides may vary from each other and from the sequences of Tables V and VI in terms of length of sequence and/or in terms of deletions, substitutions, insertions or additions of amino acids in the sequence, with consequent potential variations in the capacity for glycosylation. As noted previously, one putative allelic form of human species EPO is believed to include a methionine residue at position 126. Expectedly, naturally-occurring allelic forms of EPO-encoding DNA and cDNA sequences are also likely to occur which code for the above-noted types of allelic polypeptides or simply employ differing codons for designation of the same polypeptides as specified.

In addition to naturally-occurring allelic forms of mature EPO, the present invention also embraces other "EPO products" such as polypeptide analogs of EPO and fragments of "mature" EPO. Following the procedures of the above-noted published application by Alton, et al. (WO/83/04053) one may readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for mature EPO in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of monkey cDNA and genomic EPO genes may be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives of EPO. Such EPO products would share at least one of the biological properties of EPO but may differ in others. As examples, projected EPO products of the invention include those which are foreshortened by e.g., deletions [Asn<sup>2</sup>, des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO, [des-Thr<sup>163</sup> through Arg<sup>166</sup>]hEPO and " $\Delta$ 27—55hEPO", the latter having the residues coded for by an entire exon deleted; or which are more stable to hydrolysis (and, therefore, may have more pronounced or longer lasting effects than naturally-occurring EPO); or which have been altered to delete one or more a potential sites for glycosylation (which may result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced by, e.g., histidine or serine residues (such as the analog [His<sup>7</sup>]hEPO) and are potentially more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine (such as the analogs [Phe<sup>15</sup>]hEPO, [Phe<sup>49</sup>]hEPO, and [Phe<sup>145</sup>]hEPO) and may bind more or less readily to EPO receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within mature EPO, which fragments may possess one activity of EPO (e.g., receptor binding) and not others (e.g., erythropoietic activity). Especially significant in this regard are those potential fragments of EPO which are elucidated upon consideration of the human genomic DNA sequence of Table VI, i.e., "fragments" of the total continuous EPO sequence which are delineated by intron sequences and which may constitute distinct "domains" of biological activity. It is noteworthy that the absence of *in vivo* activity for any one or more of the "EPO products" of the invention is not wholly preclusive of therapeutic utility (see, Weiland, et al., *supra*) or of utility in other contexts, such as in EPO assays or EPO antagonism. Antagonists of erythropoietin may be quite useful in treatment of polycythemias or cases of overproduction of EPO [see, e.g., Adamson, *Hasp. Practice*, 18(12), 49—57 (1983), and Hellman, et al., *Clin. Lab. Haemat.*, 5, 335—342 (1983)].

According to another aspect of the present invention, the cloned DNA sequences described herein which encode human and monkey EPO polypeptides are conspicuously valuable for the information which they provide concerning the amino acid sequence of mammalian erythropoietin which has heretofore been unavailable despite decades of analytical processing of isolates of naturally-occurring products. The DNA sequences are also conspicuously valuable as products useful in effecting the large scale microbial synthesis of erythropoietin by a variety of recombinant techniques. Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and

5 circular plasmid DNA vectors, new and useful transformed and transfected microbial prokaryotic and eucaryotic host cells (including bacterial and yeast cells and mammalian cells grown in culture), and new and useful methods for cultured growth of such microbial host cells capable of expression of EPO and EPO products. DNA sequences of the invention are also conspicuously suitable materials for use as labelled probes in isolating EPO and related protein

10 encoding cDNA and genomic DNA sequences of mammalian species other than human and monkey species herein specifically illustrated. The extent to which DNA sequences of the invention will have use in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in humans and other mammals cannot yet be calculated. DNA sequences of the invention are expected to be useful in developing transgenic mammalian species which may serve as eucaryotic "hosts" for production of erythropoietin and erythropoietin products in quantity. See, generally,

15 Palmiter, et al., *Science*, 222(4625), 809—814 (1983).

10 Viewed in this light, therefore, the specific disclosures of the illustrative examples are clearly not intended to be limiting upon the scope of the present invention and numerous modifications and variations are expected to occur to those skilled in the art. As one example, while DNA sequences provided by the illustrative examples include monkey cDNA and genomic DNA sequences, because this application provides amino acid sequence information essential to manufacture of DNA sequence, the invention also comprehends such manufactured DNA sequences as may be constructed based on knowledge of EPO amino acid sequences. These may code for EPO (as in Example 12) as well as for EPO fragments and EPO polypeptide analogs (i.e., "EPO Products") which may share one or more biological properties of naturally-occurring EPO but not share others (or possess others to different degrees).

20 DNA sequences provided by the present invention are thus seen to comprehend all DNA sequences suitable for use in securing expression in a prokaryotic or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin, and selected from among: (a) the DNA sequences set out in Tables V and VI; (b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b). It is noteworthy in this regard, for example, that existing allelic monkey

25 and human EPO gene sequences and other mammalian species gene sequences are expected to hybridize to the sequences of Tables V and VI or to fragments thereof. Further, but for the degeneracy of the genetic code, the SCEPO and ECEPO genes and the manufactured or mutagenized cDNA or genomic DNA sequences encoding various EPO fragments and analogs would also hybridize to the above-mentioned DNA sequences. Such hybridizations could readily be carried out under the hybridization conditions described herein with respect to the initial isolation of the monkey

30 and human EPO-encoding DNA or more stringent conditions, if desired to reduce background hybridization.

35 In a like manner, while the above examples illustrate the invention of microbial expression of EPO products in the context of mammalian cell expression of DNA inserted in a hybrid vector of bacterial plasmid and viral genomic origins, a wide variety of expression systems are within the contemplation of the invention. Conspicuously comprehended are expression systems involving vectors of homogeneous origins applied to a variety of bacterial, yeast and mammalian cells in culture as well as to expression systems not involving vectors (such as calcium phosphate transfection of cells). In this regard, it will be understood that expression of, e.g., monkey origin DNA in monkey host cells in culture and human host cells in culture, actually constitute instances of "exogenous" DNA expression inasmuch as the EPO DNA whose high level expression is sought would not have its origins in the genome of the host. Expression systems of the invention further contemplate these practices resulting in cytoplasmic formation of EPO products and accumulation of

40 glycosylated and non-glycosylated EPO products in host cell cytoplasm or membranes (e.g., accumulation in bacterial periplasmic spaces) or in culture medium supernatants as above illustrated, or in rather uncommon systems such as *P. aeruginosa* expression systems (described in Gray, et al., *Biotechnology*, 2, pp. 161—165 (1984).

45 Improved hybridization methodologies of the invention, while illustratively applied above to DNA/DNA hybridization screenings are equally applicable to RNA/RNA and RNA/DNA screening. Mixed probe techniques as herein illustrated generally constitute a number of improvements in hybridization processes allowing for more rapid and reliable polynucleotide isolations. These many individual processing improvements include: improved colony transfer and maintenance procedures; use of nylon-based filters such as GeneScreen and GeneScreen Plus to allow reprobing with same filters and repeated use of the filter, application of novel protease treatments [compared, e.g., to Taub, et al. *Anal. Biochem.*, 126, pp. 222—230 (1982)]; use of very low individual concentrations (on the order of 0.025 picomole) of a large number of mixed probes (e.g., numbers in excess of 32); and, performing hybridization and post-hybridization steps under stringent temperatures closely approaching (i.e., within 4°C and preferably within 2°C away from) the lowest calculated dissociation temperature of any of the mixed probes employed. These improvements combine to provide results which could not be expected to attend their use. This is amply illustrated by the fact that mixed probe procedures involving 4 times the number of probes ever before reported to have been successfully used in even cDNA screens on messenger RNA species of relatively low abundance were successfully applied to the isolation of a unique sequence gene in a genomic library screening of 1,500,000 phage plaques. This feat was accomplished essentially concurrently with the publication of the considered opinion of Anderson, et al., *supra*, that mixed probe screening methods were "...impractical for isolation of mammalian protein genes when corresponding RNA's are unavailable.

## Claims

1. A DNA sequence for use in securing expression in a procaryotic or eucaryotic host cell of a polypeptide product having at least part of the primary structural confirmation [sic] of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells and to increase hemoglobin [sic] synthesis or iron uptake, said DNA sequence selected from the group consisting of:
  - (a) the DNA sequences set out in Tables V and VI or their complementary strands;
  - (b) DNA sequences which hybridize under stringent conditions to the protein coding regions of the DNA sequences defined in (a) or fragments thereof; and
  - (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).
- 10 2. A DNA sequence according to Claim 1 encoding human erythropoietin.
3. A cDNA sequence according to Claim 1 being a monkey species erythropoietin coding DNA sequence.
- 20 4. A DNA sequence according to Claim 3 and including the protein coding region set forth in Table V.
5. A genomic DNA sequence according to Claim 1 or 2.
- 25 6. A human species erythropoietin coding DNA sequence according to Claim 5.
7. A DNA sequence according to Claim 6 and including the protein coding region set forth in Table VI.
8. A DNA sequence according to Claim 1 or 2, covalently associated with a detectable label substance.
- 30 9. A DNA sequence according to Claim 8, wherein the detectable label is a radiolabel.
10. A single-strand DNA sequence according to Claim 8 or 9.
11. A DNA sequence according to Claim 1, coding for [Phe<sup>15</sup>]hEPO, [Phe<sup>49</sup>]hEPO, [Phe<sup>145</sup>]hEPO, [His<sup>7</sup>]hEPO, [Asn<sup>2</sup> des-Pro<sup>2</sup> through Ile<sup>6</sup>]hEPO, [des-Thr<sup>163</sup> through Arg<sup>166</sup>]hEPO, or [Δ27-55]hEPO.
12. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to any one of Claims 1, 2, 3, 6, 7 and 8, in a manner allowing the host cell to express said polypeptide product.
- 40 13. A transformed or transfected host cell according to Claim 12 which host cell is capable of glycosylating said polypeptide.
14. A transformed or transfected mammalian host cell according to Claim 13.
- 45 15. A transformed or transfected COS cell according to Claim 13.
16. A transformed or transfected CHO cell according to Claim 13.
17. A biologically functional circular plasmid or viral DNA vector including a DNA sequence according to any one of Claims 1, 2, 3, 5, 6, 7, or 11.
- 50 18. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to Claim 17.
19. A recombinant polypeptide having part or all of the primary structural conformation of human or monkey erythropoietin as set forth in Table VI or Table V or any allelic variant or derivative thereof possessing the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells to increase hemoglobin synthesis or iron uptake and characterized by being the product of eucaryotic expression of an exogenous DNA sequence and which has higher molecular weight by SDS-PAGE from erythropoietin isolated from

urinary sources.

20. A glycoprotein polypeptide according to Claim 19 having an average carbohydrate composition which differs from that of human erythropoietin isolated from urinary sources.

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21. A polypeptide according to Claim 19 or 20 wherein the exogenous sequence is a cDNA sequence.

22. A polypeptide according to Claim 19 or 20 wherein the exogenous DNA sequence is a genomic DNA sequence.

10

23. A polypeptide according to Claim 19 or 20 wherein the exogenous DNA sequence is carried on an autonomously replicating circular DNA plasmid or viral vector.

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24. A polypeptide according to any one of Claims 19 to 23 further characterized by being covalently associated with a detectable label substance.

20

25. A polypeptide according to Claim 24, wherein said detectable label is a radiolabel.

26. A polypeptide product of the expression in a eucaryotic host cell of a DNA sequence according to any of Claims 1, 2, 3, 5, 6 and 7.

25

27. A process for production of a polypeptide having at least part of the primary structural conformation of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells and to increase hemoglobin synthesis or iron uptake, which process is characterized by culturing under suitable nutrient conditions a prokaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to any of Claims 1, 2, 3, 5, 6 and 7 in a manner allowing the host cell to express said polypeptide; and optionally isolating the desired polypeptide product of the expression of the DNA sequence.

28. A process according to Claim 27, characterized by culturing a host cell of any one of Claims 12 to 16.

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29. A process according to Claim 27 or 28 for production of a polypeptide of any one of Claims 19 to 23 and 26.

30. A pharmaceutical composition comprising a polypeptide produced in accordance with the process of Claim 27, 28 or 29 and a pharmaceutically acceptable diluent, adjuvant or carrier.

35

31. A pharmaceutical composition according to Claim 30, comprising a polypeptide of any one of Claims 19 to 23 and 26.

#### Patentansprüche

40

1. DNA-Sequenz, die dafür verwendet wird, Expression in einer prokaryontischen oder eukaryontischen Wirtszelle eines Polypeptidprodukts zu erreichen, das zumindest teilweise die Primärstrukturkonformation von Erythropoietin aufweist, um den Besitz der biologischen Eigenschaft zu ermöglichen, Knochenmarkszellen zu veranlassen, die Produktion von Retikulozyten und roten Blutkörperchen zu steigern und Hämoglobinsynthese oder Eisenaufnahme zu steigern, wobei besagte DNA-Sequenz aus der Gruppe ausgewählt ist, die aus

(a) den in den Tabellen V und VI dargelegten DNA-Sequenzen oder deren Komplementärsträngen;  
(b) DNA-Sequenzen, die unter stringenten Bedingungen zu den proteincodierenden Bereichen der in (a) definierten DNA-Sequenzen oder Fragmenten derselben hybridisieren; und  
(c) DNA-Sequenzen, die ohne die Entartung des genetischen Codes zu den in (a) und (b) definierten DNA-Sequenzen hybridisieren würden;

besteht.

55 2. DNA-Sequenz nach Anspruch 1, die Human-Erythropoietin codiert.

3. cDNA-Sequenz gemäß Anspruch 1, die ein Erythropoietin einer Affenart codiert.

4. DNA-Sequenz nach Anspruch 3, die den in Tabelle V dargestellten proteincodierenden Bereich einschließt.
5. Genomische DNA-Sequenz nach Anspruch 1 oder 2.
5. DNA-Sequenz nach Anspruch 5, die ein Erythropoetin einer menschlichen Spezies codiert.
7. DNA-Sequenz nach Anspruch 6, die den in Tabelle VI dargestellten proteincodierenden Bereich einschließt.
8. DNA-Sequenz nach Anspruch 1 oder 2, die kovalent mit einer nachweisbaren Markierungssubstanz verbunden ist.
10. DNA-Sequenz nach Anspruch 8, dadurch gekennzeichnet, daß die nachweisbare Markierung eine radioaktive Markierung ist.
10. Einzelsträngige DNA-Sequenz nach Anspruch 8 oder 9.
15. DNA-Sequenz nach Anspruch 1, die für [Phe<sup>15</sup>]hEPO, [Phe<sup>49</sup>]hEPO, [Phe<sup>145</sup>]hEPO, [His<sup>7</sup>]hEPO, [Asn<sup>2</sup> des - Pro<sup>2</sup> bis Ile<sup>6</sup>]hEPO, [des - Thr<sup>163</sup> bis Arg<sup>166</sup>]hEPO oder [Δ27-55]hEPO codiert.
20. Prokaryotische oder eukaryotische Wirtszelle, transformiert oder transfiziert mit einer DNA-Sequenz nach einem der Ansprüche 1, 2, 3, 6, 7 und 8, in einer Weise, die es der Wirtszelle erlaubt, besagtes Polypeptidprodukt zu exprimieren.
25. Transformierte oder transfizierte Wirtszelle nach Anspruch 12, dadurch gekennzeichnet, daß die Wirtszelle in der Lage ist, besagtes Polypeptid zu glykosylieren.
14. Transformierte oder transfizierte Säugetier-Wirtszelle nach Anspruch 13.
15. Transformierte oder transfizierte COS-Zelle nach Anspruch 13.
30. Transformierte oder transfizierte CHO-Zelle nach Anspruch 13.
17. Biologisch funktioneller Zirkular-Plasmid- oder Virus-DNA-Vektor, der eine DNA-Sequenz nach einem der Ansprüche 1, 2, 3, 5, 6, 7 oder 11 einschließt.
35. Prokaryotische oder eukaryotische Wirtszelle, die mit einem DNA-Vektor nach Anspruch 17 stabil transformiert oder transfiziert ist.
40. Rekombinantes Polypeptid, das einen Teil oder die Gesamtheit der Primärstrukturkonformation von Human- oder Affen-Erythropoetin, wie dargestellt in Tabelle VI oder Tabelle V, oder irgendeine allelische Variante oder ein Derivat desselben aufweist, das die biologische Eigenschaft besitzt, Knochenmarkszellen zu veranlassen, die Produktion von Retikulozyten oder roten Blutkörperchen zu steigern und Hämoglobinsynthese oder Eisenaufnahme zu steigern, dadurch gekennzeichnet, daß es das Produkt eukaryotischer Expression einer exogenen DNA-Sequenz ist und welches gemäß SDS-PAGE ein höheres Molekulargewicht hat als Erythropoetin, das aus Urin-Quellen isoliert ist.
45. Glycoprotein-Polypeptid nach Anspruch 19, das eine mittlere Kohlenhydratzusammensetzung aufweist, die sich von derjenigen von Human-Erythropoetin unterscheidet, das aus Urin-quellen isoliert ist.
50. Polypeptid nach Anspruch 19 oder 20, dadurch gekennzeichnet, daß die exogene DNA-Sequenz eine cDNA-Sequenz ist.
22. Polypeptid nach Anspruch 19 oder 20, dadurch gekennzeichnet, daß die exogene DNA-Sequenz eine genomische DNA-Sequenz ist.
55. Polypeptid nach Anspruch 19 oder 20, dadurch gekennzeichnet, daß die exogene DNA-Sequenz auf einem autonom replizierenden Zirkular-DNA-Plasmid- oder Virus-Vektor sitzt.
24. Polypeptid nach einem der Ansprüche 19 bis 23, weiter dadurch gekennzeichnet, daß es kovalent mit einer nach-

weisbaren Markierungssubstanz verbunden ist.

25. Polypeptid nach Anspruch 24, dadurch gekennzeichnet, daß besagte nachweisbare Markierung eine radioaktive Markierung ist.

5 26. Polypeptidprodukt der Expression einer eukaryontischen Wirtszelle einer DNA-Sequenz nach einem der Ansprüche 1, 2, 3, 5, 6 und 7.

10 27. Verfahren zur Herstellung eines Polypeptids, das zumindest teilweise die Primärstrukturkonformation von Erythropoietin aufweist, um den Besitz der biologischen Eigenschaft zu ermöglichen, Knochenmarkszellen zu veranlassen, die Produktion von Retikulozyten und roten Blutkörperchen zu steigern und Hämoglobinsynthese oder Eisenaufnahme zu steigern, dadurch gekennzeichnet, daß eine prokaryontische oder eukaryontische Wirtszelle, die mit einer DNA-Sequenz nach einem der Ansprüche 1, 2, 3, 5, 6 und 7 derart transformiert oder transfiziert worden ist, daß es der Wirtszelle ermöglicht ist, besagtes Polypeptid zu exprimieren, unter geeigneten Nährbedingungen kultiviert wird; und daß fakultativ das gewünschte Polypeptidprodukt der Expression der DNA-Sequenz isoliert wird.

15 28. Verfahren nach Anspruch 27, dadurch gekennzeichnet, daß eine Wirtszelle nach einem der Ansprüche 12 bis 16 kultiviert wird.

20 29. Verfahren nach Anspruch 27 oder 28 zur Produktion eines Polypeptids nach einem der Ansprüche 19 bis 23 und 26.

30. Pharmazeutische Zusammensetzung, die ein Polypeptid, das gemäß dem Verfahren nach Anspruch 27, 28 oder 29 produziert ist, und ein pharmazeutisch annehmbares Verdünnungsmittel, Adjuvans oder Trägermittel umfaßt.

25 31. Pharmazeutische Zusammensetzung nach Anspruch 30, die ein Polypeptid nach einem der Ansprüche 19 bis 23 und 26 umfaßt.

30 **Revendications**

1. Séquence d'ADN utilisable pour garantir l'expression, dans une cellule hôte procaryote ou eucaryote, d'un produit polypeptidique ayant au moins une partie de la conformation structurelle primaire de celle de l'érythropoïétine pour lui permettre de posséder la propriété biologique de provoquer, par les cellules de la moelle osseuse, l'augmentation de la production des réticulocytes et des globules rouges, et l'augmentation de la synthèse d'hémoglobine ou du captage de fer, ladite séquence d'ADN étant choisie dans le groupe constitué de :

35 a) les séquences d'ADN indiquées dans les tableaux V et VI ou leurs brins complémentaires ;  
b) des séquences d'ADN qui s'hybrident dans des conditions strictes aux régions de codage de protéine des séquences d'ADN définies dans (a) ou des fragments de celles-ci; et  
c) des séquences d'ADN qui, n'était la dégénérescence du code génétique, s'hybrideraient aux séquences d'ADN définies dans (a) et (b).

40 2. Séquence d'ADN selon la revendication 1, codant l'érythropoïétine humaine.

45 3. Séquence d'ADNc selon la revendication 1, qui est une séquence d'ADN codant l'érythropoïétine de singe.

4. Séquence d'ADN selon la revendication 3 et incluant la région de codage de protéine indiquée dans le tableau V.

50 5. Séquence d'ADN génomique selon la revendication 1 ou 2.

6. Séquence d'ADN codant l'érythropoïétine humaine selon la revendication 5.

7. Séquence d'ADN selon la revendication 6 et incluant la région de codage de protéine indiquée dans le tableau VI.

55 8. Séquence d'ADN selon la revendication 1 ou 2, associée de façon covalente à une substance de marquage détectable.

9. Séquence d'ADN selon la revendication 8, dans laquelle le marqueur détectable est un radiomarqueur.

10. Séquence d'ADN simple brin selon la revendication 8 ou 9.
11. Séquence d'ADN selon la revendication 1 codant pour [Phe<sup>15</sup>]EPO, [Phe<sup>49</sup>]hEPO, [Phe<sup>145</sup>]hEPO, [His<sup>7</sup>]hEPO, [Asn<sup>2</sup> des-Pro<sup>2</sup> à Ile<sup>6</sup>]hEPO, [des-Thr<sup>163</sup> à Arg<sup>166</sup>]hEPO, ou [Δ27-55]hEPO.
- 5 12. Cellule hôte procaryote ou eucaryote transformée ou transfectée par une séquence d'ADN selon l'une quelconque des revendications 1, 2, 3, 6, 7 et 8, d'une façon permettant à la cellule hôte d'exprimer ledit produit polypeptidique.
- 10 13. Cellule hôte transformée ou transfectée selon la revendication 12, laquelle cellule hôte est capable de glycosyler ledit polypeptide.
14. Cellule hôte de mammifère transformée ou transfectée selon la revendication 13.
15. Cellule COS transformée ou transfectée selon la revendication 13.
16. Cellule CHO transformée ou transfectée selon la revendication 13.
17. Vecteur d'ADN viral ou de plasmide circulaire biologiquement fonctionnel, incluant une séquence d'ADN selon une quelconque des revendications 1, 2, 3, 5, 6, 7 ou 11.
- 20 18. Cellule hôte procaryote ou eucaryote transformée ou transfectée de façon stable par un vecteur d'ADN selon la revendication 17.
- 25 19. Polypeptide recombinant ayant tout ou partie de la conformation structurelle primaire de l'érythropoïétine humaine ou de singe, comme indiquée dans le tableau VI ou le tableau V, ou tout variant allélique ou dérivé de celui-ci possédant la propriété biologique de provoquer, par les cellules de la moelle osseuse, l'augmentation de la production des réticulocytes et des globules rouges et l'augmentation de la synthèse d'hémoglobine ou du captage de fer, et caractérisé en ce qu'il est le produit de l'expression eucaryote d'une séquence d'ADN exogène et qui présente un poids moléculaire plus élevé par SDS-PAGE à partir de l'érythropoïétine isolée de sources urinaires.
- 30 20. Polypeptide de glycoprotéine selon la revendication 19 ayant une composition moyenne de carbohydrate qui diffère de celle de l'érythropoïétine humaine isolée de sources urinaires.
21. Polypeptide selon la revendication 19 ou 20, dans lequel la séquence exogène est une séquence d'ADNc.
- 35 22. Polypeptide selon la revendication 19 ou 20, dans lequel la séquence d'ADN exogène est une séquence d'ADN génomique.
23. Polypeptide selon la revendication 19 ou 20, dans lequel la séquence d'ADN exogène est portée sur un vecteur viral ou de plasmide d'ADN circulaire se répliquant de façon autonome.
- 40 24. Polypeptide selon l'une quelconque des revendications 19 à 23, caractérisé de plus en étant associé de façon covalente à une substance de marquage détectable.
25. Polypeptide selon la revendication 24, dans lequel ledit marqueur détectable est un radiomarqueur.
26. Produit polypeptidique de l'expression, dans une cellule hôte eucaryote, d'une séquence d'ADN selon une quelconque des revendications 1, 2, 3, 5, 6 et 7.
- 50 27. Procédé pour produire un polypeptide ayant au moins une partie de la conformation structurelle primaire de l'érythropoïétine pour lui permettre de posséder la propriété biologique de provoquer, par les cellules de la moelle osseuse, l'augmentation de la production des réticulocytes et des globules rouges, et l'augmentation de la synthèse d'hémoglobine ou du captage de fer, lequel procédé est caractérisé par la mise en culture, dans des conditions nutritives appropriées, d'une cellule hôte procaryote ou eucaryote transformée ou transfectée avec une séquence d'ADN selon une quelconque des revendications 1, 2, 3, 5, 6 et 7 d'une manière permettant à la cellule hôte d'exprimer ledit polypeptide et, éventuellement, l'isolement du produit polypeptidique souhaité de l'expression de la séquence d'ADN.

28. Procédé selon la revendication 27, caractérisé par la mise en culture d'une cellule hôte selon l'une quelconque des revendications 12 à 16.
29. Procédé selon la revendication 27 ou 28 pour produire un polypeptide selon l'une quelconque des revendications 5 19 à 23 et 26.
30. Composition pharmaceutique comprenant un polypeptide produit conformément au procédé de la revendication 27, 28 ou 29 et un support, adjuvant ou diluant pharmaceutiquement acceptable.
- 10 31. Composition pharmaceutique selon la revendication 30, comprenant un polypeptide selon une quelconque des revendications 19 à 23 et 26.

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FIG. 1 Comparison of Recombinant Human &amp; Monkey EPO in Radioimmunoassay

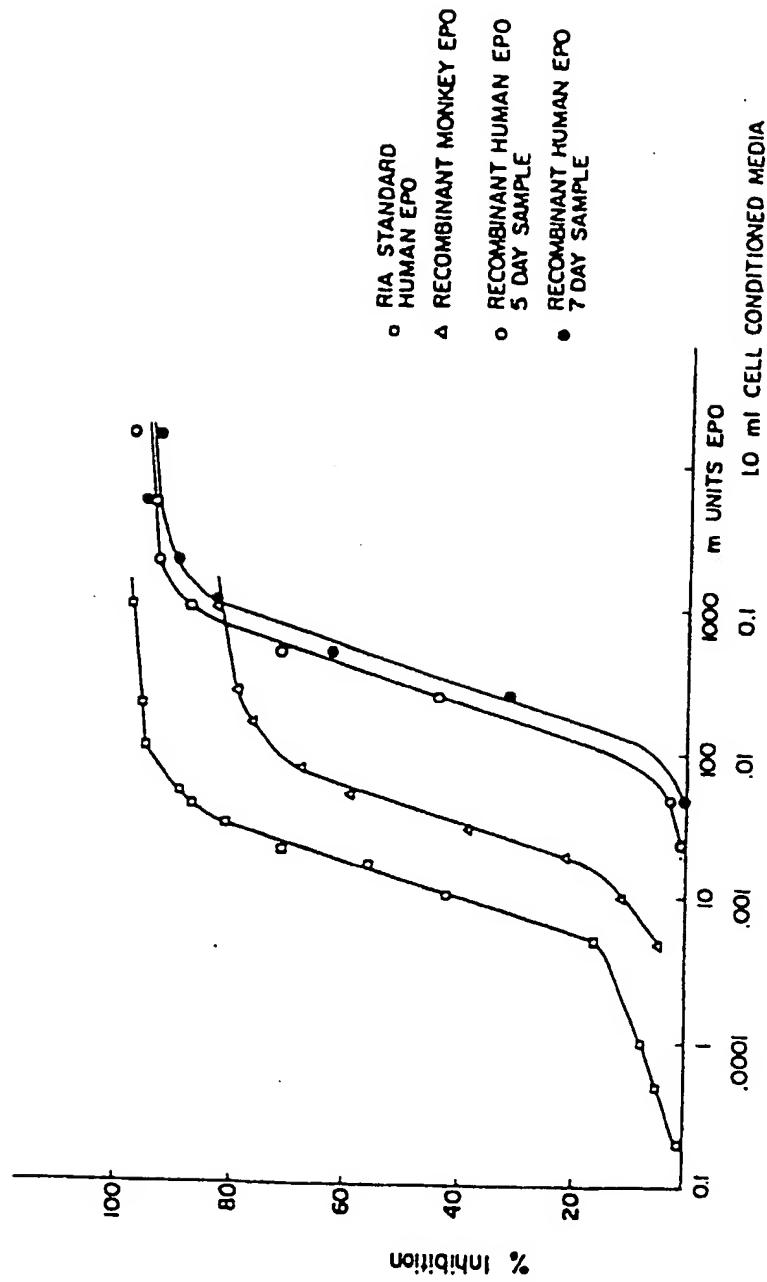


FIG. 2

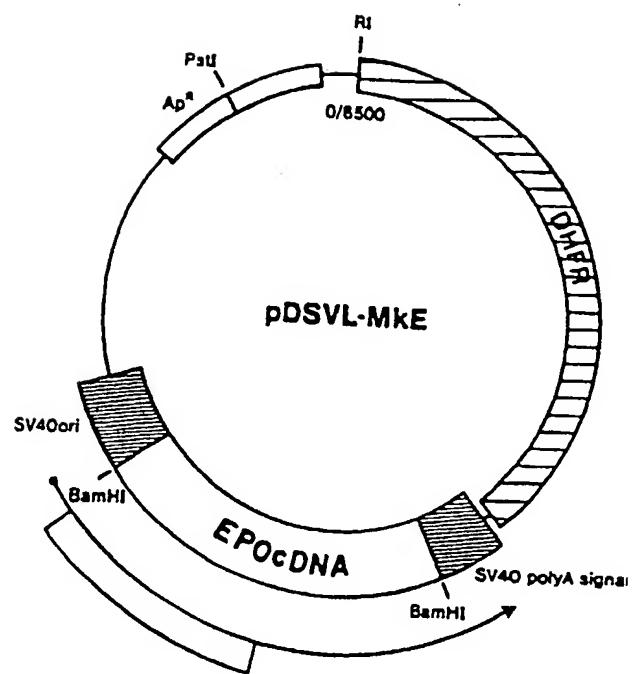


FIG. 3

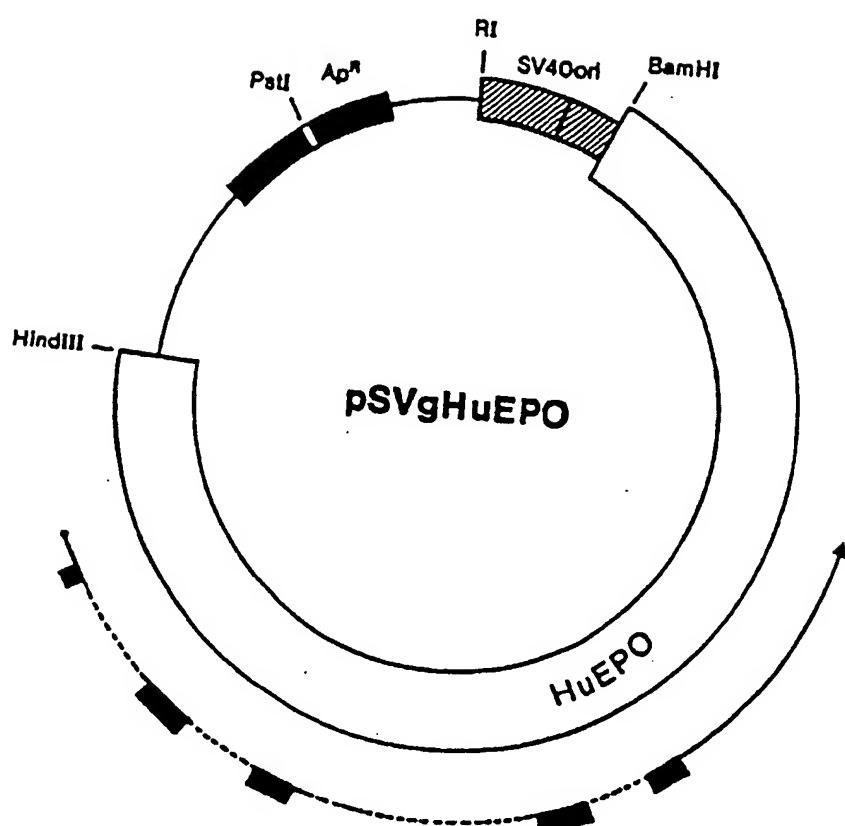


FIG. 4

